06-27/07-18 PCR linearization attempts for Gibson, pRS415-GAL/pJR18

Project: Promoter
Authors: Pihla Savola
Dates: 2016-06-27 to 2016-07-18

MONDAY, 6/27

Linearizing pRS415-GAL for use in stress promoter Gibson Assembly. Using attached protocol.

Primers HH1 and HH2 were resuspended and diluted to a final concentration of 50 uM using the attached protocol.
PCR reaction was set up as follows:

- H2O - 33 uL
- 5X Phusion HF buffer - 10 uL
- 2 nM dNTPs - 5 uL
- Forward primer 50 uM - 0.5 uL
- Reverse primer 50 uM - 0.5 uL
- Template DNA - 0.5 uL (112.4 uM -> 1:50 dilution -> 2.248 uM -> 0.5 uL for 1.124 ng template)
- Phusion DNA Polymerase - 0.5 uL

PCR cycling condition was as follows

- 1. Initial denaturation: 98 C, 30 s
- 2. Denaturation 98 C, 10 s
- 3. Annealing 55 C, 30 s
- 4. Extension 72 C, 2 min -> cycle back to 2, 5 cycles
- 5. Denaturation 98 C, 10 s
- 6. Annealing 58 C, 30 s
- 7. Extension 72 C, 2 min -> cycle back to 5, 30 cycles
- 8. Final extension 72 C, 10 min
- Hold afterwards at 4 C.

To vizualise the results of the PCR, gel electrophoresis was conducted using a 0,7 % agarose gel.

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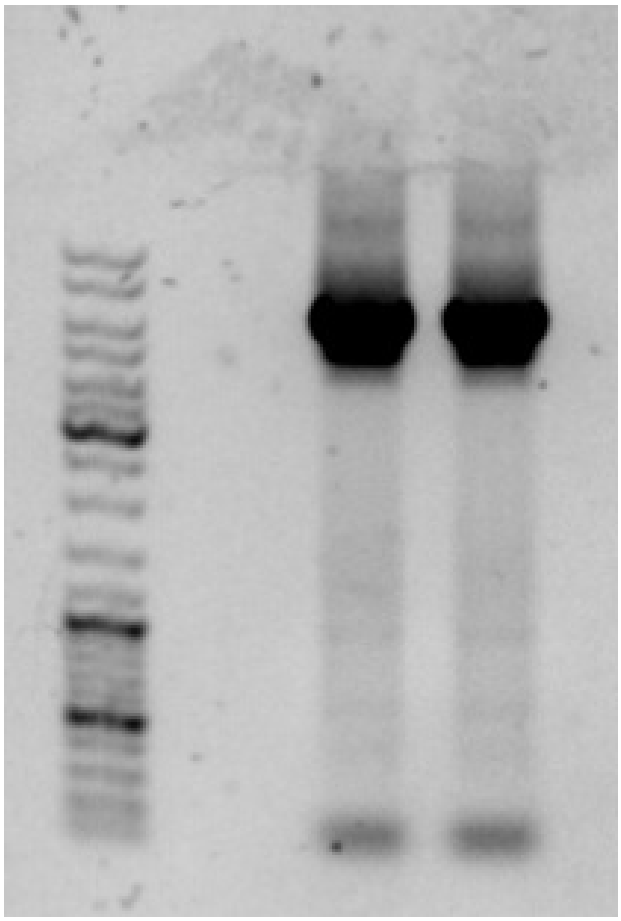

Samples were run on the gel and the identified band was cut out and gel purified with Macherey-Nagel PCR and Gel extarction kit (+protocol) in two tubes (samples A and B, which both contain the same material but in different concentrations).

Elution was done into three fractions. Final DNA concentrations of samples was measured from the eluted fraction and recorded in Table 1.

Table1

	A	B	C
1	Sample A	Conc. of DNA (ug/mL)	Average
2	1 (30 ul)	10.4	
3	2 (20 ul)	8.8	
4	3 (20 ul)	3.7	7.6333333333
5	Sample B		
6	1 (30 ul)	8	
7	2 (20 ul)	8.7	
8	3 (20 ul)	3.1	6.6

TUESDAY, 6/28

More pRS415-GAL plasmid was prepared for stress promoter assembly using attached protocol. PCR reaction without template is run in parallel as a control.

A mastermix for the two reactions was prepared:
H2O - 33.6 uL
5X Phusion HF buffer - 10 uL
2 nM dNTPs - 5 uL
Forward primer 50 uM - 0.2 uL
Reverse primer 50 uM - 0.2 uL

Mastermix was divided to solutions with volumes 29,4 uL (AKR2) and 19.6 uL (negative control). 0.3 uL phusion DNA polymerase and 0.3 uL template DNA was added to AKR2 and 0.2 uL of phusion DNA polymerase and 0.2 uL water H2O was added to control sample.

PCR was run with parameters:

- 1. Initial denaturation: 98 C, 30 s
- 2. Denaturation 98 C, 10 s
- 3. Annealing 55 C, 30 s
- 4. Extension 72 C, 1 min 40 seconds -> cycle back to 2, 5 cycles
- 5. Denaturation 98 C, 10 s
- 6. Annealing 58 C, 30 s
- 7. Extension 72 C, 1 min 40 seconds -> cycle back to 5, 30 cycles
- 8. Final extension 72 C, 10 min
- Hold afterwards at 4 C.

Gel electrophoresis was executed with 0.7% agarose gel with todays samples (2.5uL sample+ 0.5 uL dye), yestedays PCR sample (PSR1, 2.5 uL sample+0.5uL dye) and the DNA template (10 uL sample+ 2 uL dye, to visualize the results of the PCR. However, no band was not observed in todays PCR (AKR2). Nevertheless, the band from yesterdays PCR sample (PSR1) was observed above 6000 kb mark.

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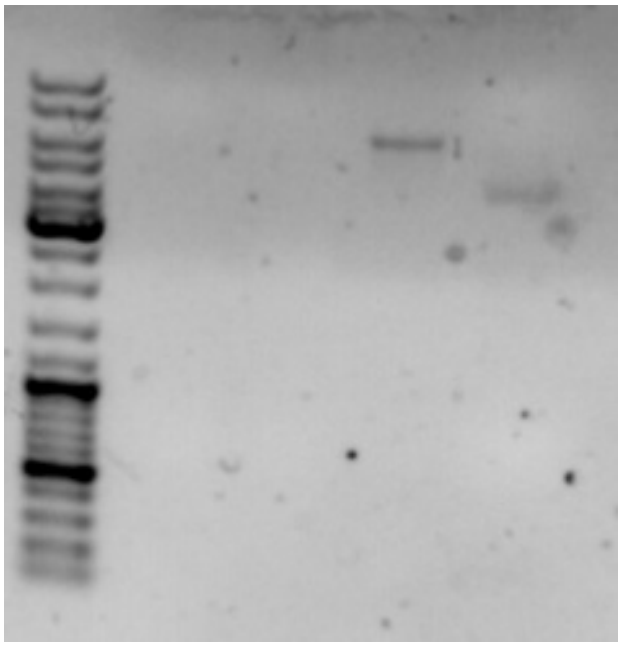


(From left; marker, empty lanes AKR1, PSR1, template)

THURSDAY, 6/30

PCR was prepared to linearize more pRS415-GAL:

Master Mix

	A	B	C
1		Volume (ul)	Final concentration
2	H2O	37	
3	10 mM dNTP	1	0.2 mM
4	5x Phusion HF buffer	10	1x
5	Forward primer	0.5	0.5 uM
6	Reverse primer	0.5	0.5 uM
7	Phusion DNA polymerase	0.5	
8	Total	49.5	
9			
10	Template (only to sample tube)	0.3	0.674 ng

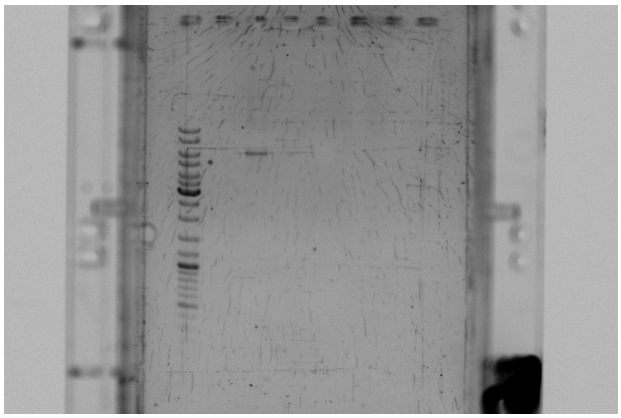
From the total volume of Master Mix, 30 ul was transferred to the 'sample' tube and the rest (29.5 ul) was kept as negative control. Nothing else was added to the negative control.

PCR program settings were same as on 27.6. The extension time was increased to 2 min from 1'40" (the previous PCR run on 28.6).

Gel electrophoresis

5 ul sample + 1 ul loading dye = 6 ul
5 ul DNA ladder

SSR1.tif



Thin band slightly above ~6000 bp observed in SSR1 sample.
No bands observed in negative control.

FRIDAY, 7/8

PCR for pJR18 backbone linearization with phusion hifi II hot start.

Switch from pRS415-GAL to pJR18, because GeneArt had probelms with cloning into pRS415-GAL template that we sent so there might be problems with it and we don't know what they are so better not to use it probably. We sent some pJR18 template to GeneArt instead of pRS415-GAL (after verifying on the gel that it didn't produce extra bands, see 07-06 Verifying pJR18 template). pJR18 is the pRS415-GAL plasmid backbone with a ~700bp insert in the multiple cloning site, inserted with SpeI and XhoI restriction enzymes.

Table2

	A	B	C	D
1		reaction	control	master mix (49ul and 19,6ul)
2	component	50ul	20ul	
3	water	23	9,2	32,2
4	master mix	25	10	35
5	forward primer HH1	0,5	0,2	0,7
6	reverse primer HH2	0,5	0,2	0,7
7	template DNA (1,12 ng/ul)	1	0,4	

Order in picture: ladder, control, plasmid PCR, ligation venus+pJR17, ligation venus+pJR18 (ligations for Venus YFP cloning, to check ligations on gel)

Näyttökuva 2016-07-20 kello 14.24.16.png

Result: weird and faint band in PCR in totally wrong place, about 3000bp. For ligation product analysis, SDS needed in loading dye.

MONDAY, 7/11

PCRs were prepared with both normal Phusion polymerase (used earlier in first, working PCRs) and Phusion Hotstart, which produced the wrong band (7/8). PCRs were prepared for both pJR18 and pRS415-GAL, in case that was the reason for the PCR failing yesterday (earlier, we had used the normal Phusion and pRS415-GAL and it had worked)

Hot start:

Table3

	A	B	C	D	E
1		pJR18	pRS415	control	master mix (9,8ul to each)
2	total volume	10ul	10ul	10ul	29,4ul
3	H2O	4,6ul	4,6ul	4,6ul	13,8ul
4	master mix	5,0ul	5,0ul	5,0ul	15ul
5	forward primer (50uM)	0,1ul	0,1ul	0,1ul	0,3ul
6	reverse primer (50uM)	0,1ul	0,1ul	0,1ul	0,3ul
7	template (1ng/ul)	0,2ul	0,2ul	0,2ul (water)	

Phusion:

Table4

	A	B	C	D	E	F
1		pJR18	pRS415	control	master mix (9,8ul to each)	
2	total volume	10	10	10	21,9	
3	H2O	7,3	7,3	7,3	21,9	
4	10mM dNTPs	0,2	0,2	0,2	0,6	
5	forward primer (50uM)	0,1	0,1	0,1	0,3	
6	reverse primer (50uM)	0,1	0,1	0,1	0,3	
7	template (1ng/ul)	0,2	0,2	0,2 (water)	-	
8	5xPhusion HF buffer	2,0	2,0	2,0	6,0	
9	Phusion polymerase	0,1	0,1	0,1	0,3	

Both mixes had to wait a while before they were put into Thermocycler (because normal machine was reserved). We also had to use old thermocycler.

Order in picture: Marker, Phusion: pJR18, pRS415, control, HOT START: pJR18, pRS415, control,

Näyttökuva 2016-07-25 kello 9.27.22.png

Result: Nothing with Phusion, same weird band with HOT START in wrong place for both templates, so most likely the problem is not with the template: further tests will be made with pJR18

TUESDAY, 7/12

New dilutions (1ng/ul) from pRS415 and pJR18 were made and stored in freezer.

WEDNESDAY, 7/13

Testing again,, and this time also trying more template.

Hot Start:

Table5

	A	B
1		pJR18
2	total volume	50ul
3	H2O	22ul
4	master mix	25ul
5	forward primer (50uM)	0,5ul
6	reverse primer (50uM)	0,5ul
7	template (1ng/ul)	2ul

Phusion:

Table6

	A	B	C	D
1		pJR18 (with two template amounts)	control	Master mix (48/48/9,6)
2	total volume	50	10	105,6
3	H2O	35,5	7,1	78,1
4	10mM dNTPs	1,0	0,2	2,2
5	forward primer (50uM)	10	2	22
6	reverse primer (50uM)	0,5	0,1	1,1
7	Phusion polymerase	0,5	0,4	1,1
8	5xPhusion HF buffer	0,5	0,1	1,1
9	template (1ng/ul)	2ul/1ul+1ul water	0,4 water	-

Order in gel: Marker, phusion 2ng template, phusion 1ng template, hot start, negative control

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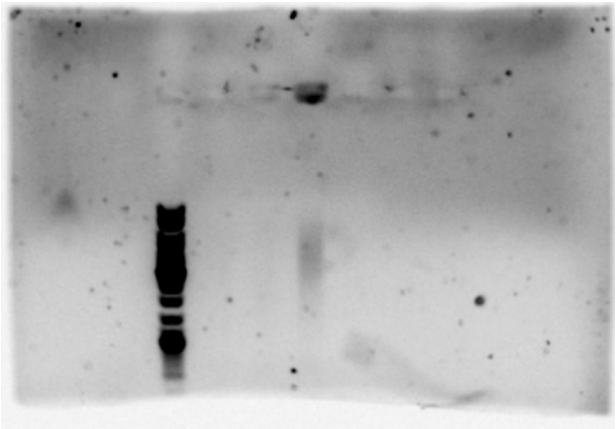

Result: Same as before.

THURSDAY, 7/14

Attempts to linearize pJR018 plasmid with PCR for Gibson assembly with stress promoters continue...

This time trting Kapa HiFi HotStart Readymix. Reaction was set up as follows, for a 50 uL reaction with pJR18 as template, and a 25 uL negative control with half the amounts with water instead of DNA as template.

Table7

	A	B	C
1	Component	50 uL rxn	Final conc.
2	Water	21 uL	N/A
3	2X ReadyMix	25.0 uL	1X
4	10 uM FW primer	1.5 uL	0.3 uM
5	10 uM RV primer	1.5 uL	0.3 uM
6	Template DNA	1 ng (1 uL)	0.02 ng/uL

PCR conditions were as follows:

Table8

	A	B	C	D
1	Step	Temperature	Duration	Cycles
2	Initial denaturation	95 C	3 min	1
3	Denaturation	98 C	20 sec	25
4	Annealing	55 C	15 sec	
5	Extension	72 C	3 min 30 sec	
6	Final extension	72 C	7 min	1
7	Hold	4 C	Infinity	

To visualize PCR results, 5 uL of PCR reaction was combined with 1 uL 6X loading dye and run on a 0.7 % agarose gel.
A band was seen around the 100bp marker, but not on the negative control.

A new PCR was set up with the following changes to the protocol;

1. A new different dilution of template was used; the volume was the same but the dilution included 4.9 ng of DNA. (The plasmid concentration was re-measured with Nanodrop; originally measured was 50,3 ng/uL, the new measurement was 49 ng/uL)
2. Annealing temperature was reduced to 50 C.

The used template dilution was checked for degradation; restriction digest was conducted:

H2O to a total volume of 20 uL
0.5 uL BcuI (SpeI)
0.5 uL XhoI
2 uL FastDigest Buffer 10X
500 ng DNA

This was digested for 30 min.

Agarose gel electrophoresis was conducted on a 0.7 % gel, and the following samples were run:

1. PCR product; 10 uL
2. pJR018 plasmid restriction digest, 10 uL;
3. Undigested pJR018 plasmid; 5 uL (245ng)

The gel appeared partly smeared when imaged, possibly due to gel shifting during the run (the gel was loose in the holder, so some of the quite full wells might have also spilled). Additionally, the dna marker ladder was only visible as a smear on the bottom of the gel. For this reason, a new gel was run the following morning; samples were stored for 14 h in +4 C.
Smeared gel below:

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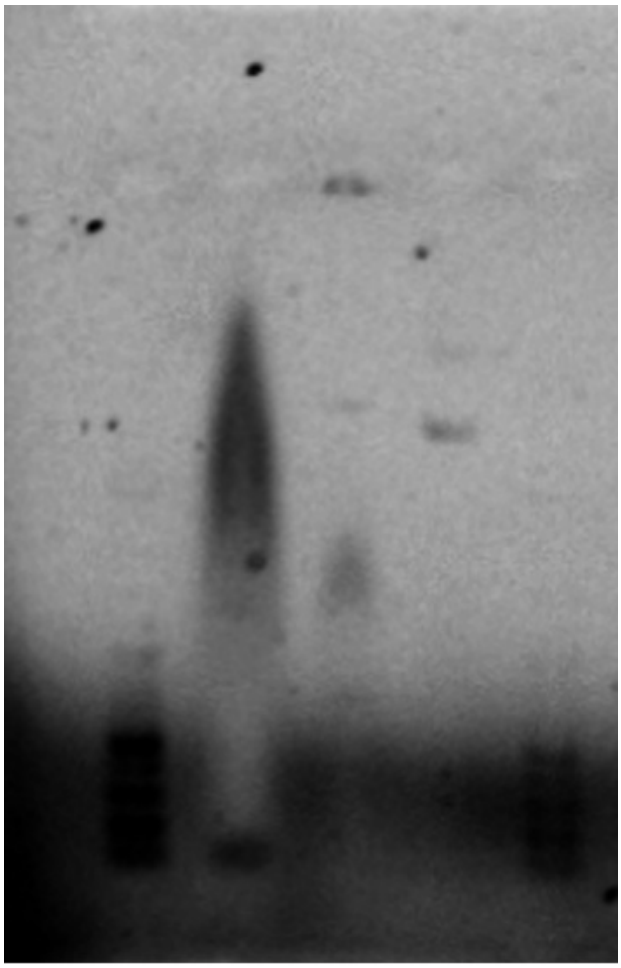

(From left: DNA ladder, PCR product, BcuI/XhoI restriction of pJR18, uncut pJR18 plasmid, DNA ladder)

FRIDAY, 7/15

The new gel was imaged (PCR in both of the 2 first wells),

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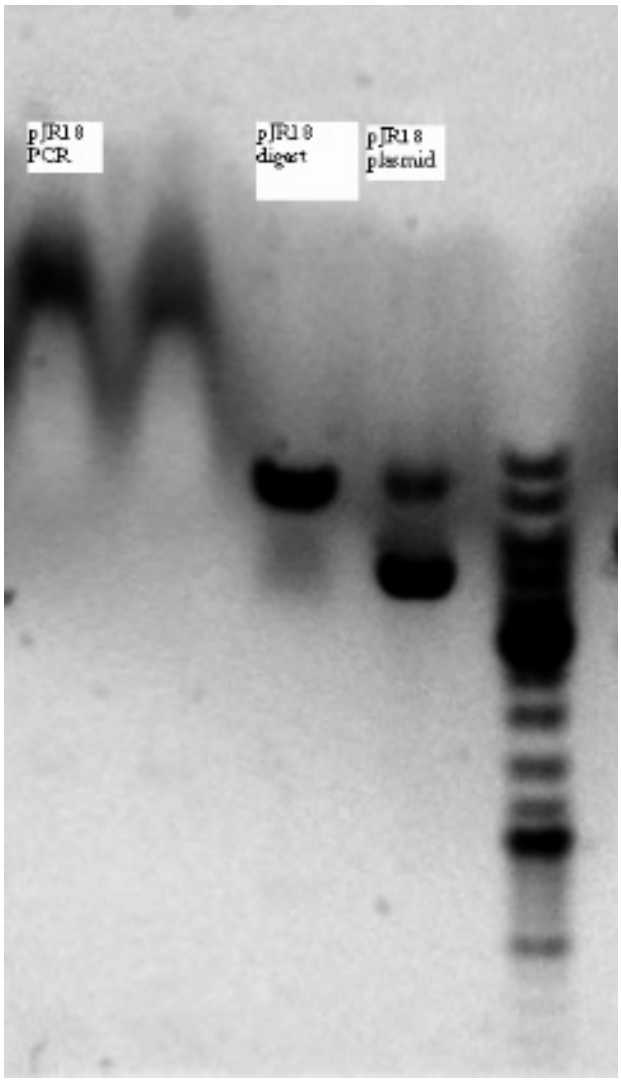

(From left: PCR product, BcuI (=SpeI)/XhoI restriction of pJR18, uncut pJR18 plasmid)

The image shows that template digestion was incomplete. The smeared PCR product was still rerun on the gel, this time switching to loading dye containing SDS (Purple loading dye). All further gels in this entry were run using purple loading dye.

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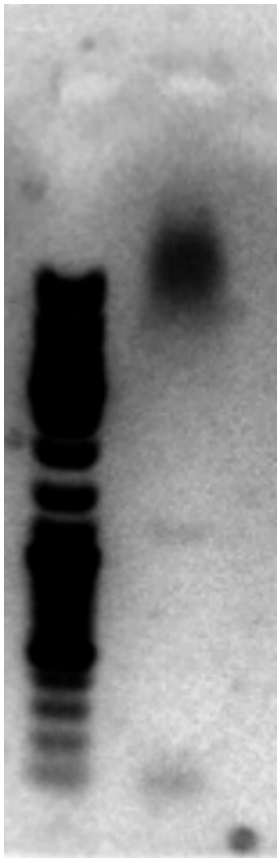

As seen above, this time a clearer result was obtained, and the PCR appeared to have produced mainly wrong products, although it looked unclear whether there might be some of the correct product as well. To confirm the results, the same PCR was redone with a different template aliquot (507 ng/uL -> diluted 1:10 to a total volume of 20 uL, and again 1:10 to a 20 uL volume for a final concentration of 5.1 ng/uL, which was checked still on nanodrop. 1 uL (5.1 ng) of this dilution was used for the PCR, which was done as previously).

As the template checks were not that clear on the previous gel, a new template sample (5 uL of 50.7ng/uL solution, a 1:10 dilution of the 507.5 ng/uL solution) and a new restriction digest were run on the gel, this time with restriction enzymes XhoI and XbaI but otherwise as before.

PCR products were run on the gel, and clear bands of incorrect PCR products were observed.

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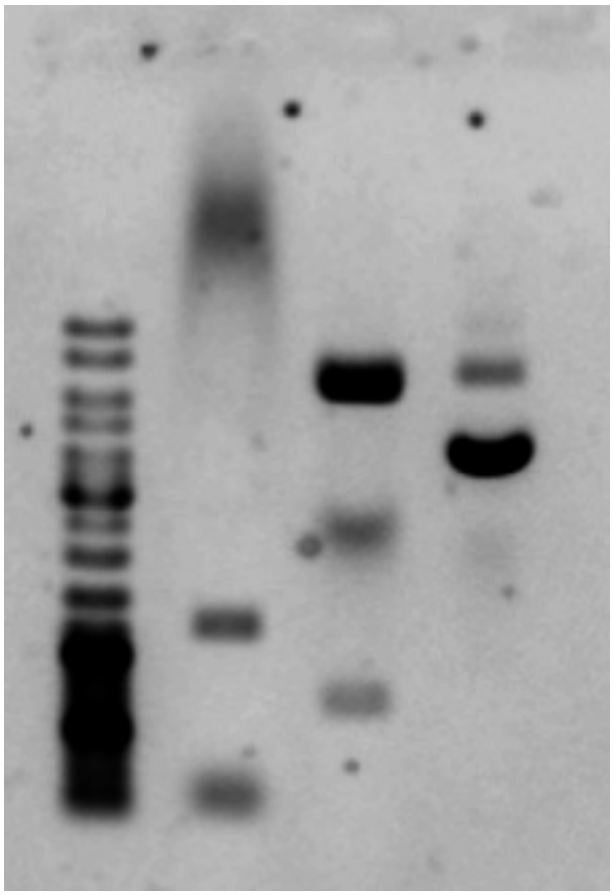

(from left; marker, PCR, digestion, undigested template)

An additional, incorrect band also seems to appear in the restriction digest of the template; the correct bands appear at 600 bp and 7000 bp. Because this band was not observed earlier - when not using XbaI - the incorrect band and XbaI's role in it was further investigated.

To troubleshoot the incorrect band, restriction digests of the pJR18 template were conducted with the following restriction enzymes and restriction enzyme combinations, according to the attached protocol;

1. SpeI, and SpeI + XhoI combination
2. XbaI, and XbaI + XhoI combination (using Biomolecular materials group common stock XbaI)
3. XbaI, and XbaI + XhoI combination (using Molecular biotechnology group common stock XbaI)

After an 30 minute incubation at 37 C, 10 uL samples of the restriction digests were run on a gel. The incorrect band was observed with all the samples.

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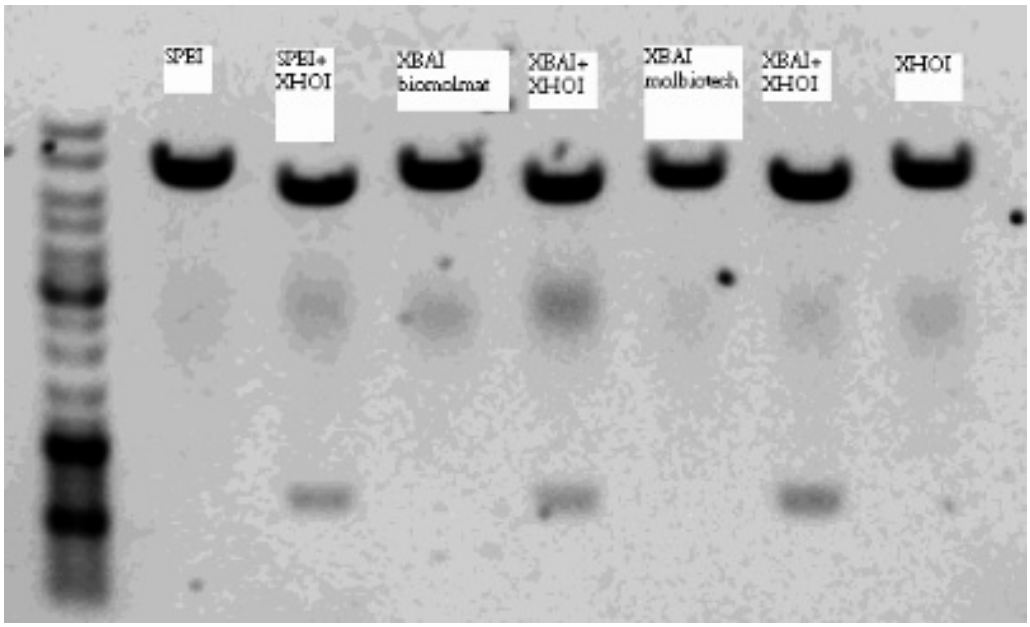

(from left: SpeI, SpeI+XhoI, XbaI(biomolmat), XbaI+XhoI, XbaI(molbiotech), XbaI+XhoI, XhoI)

MONDAY, 7/18

As the extra band semed to appear using all restriction enzyme combinations, the problem was further troubleshooted by conducting the same restriction digests in parallel using pRS415-GAL plasmid template (112 ng/uL solution; used 4.5 uL of this for each digest). After 15 min of incubation with the enzymes, and gel electrophoresis, the following image was obtained:

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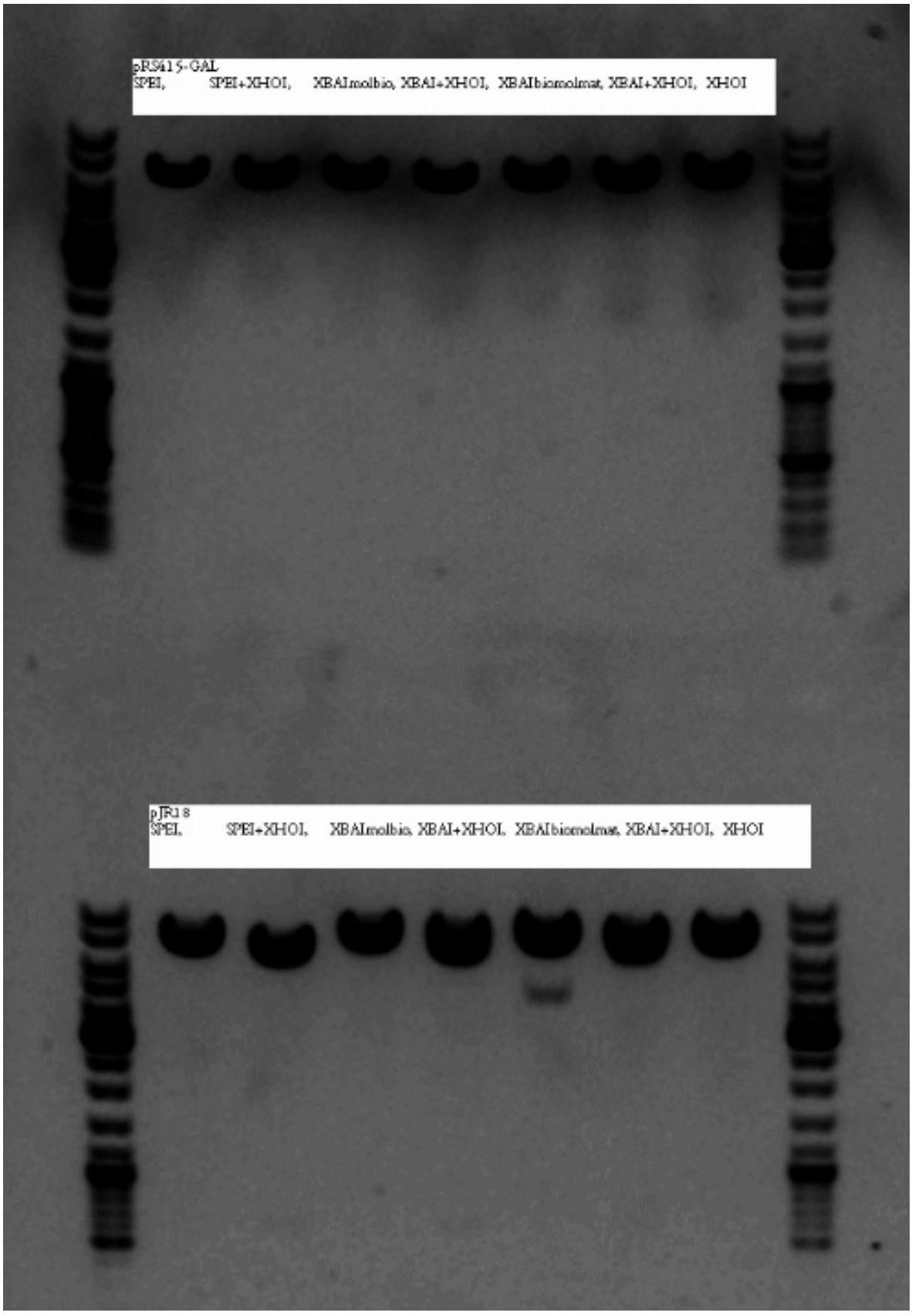

(from left: top row pRS415-GAL with SpeI, SpeI+XhoI, XbaI(molbiotech), XbaI+XhoI, XbaI(biomolmat), XbaI+XhoI, XhoI, bottom row pJR18 and same order with enzymes)

The smaller band on pJR18 with XbaI (biomolmat) appears to correspond to uncut plasmid. Although no clear bands can be observed around 700 bp for the double digests, the double digest would appear to be succesful when comparing the size of the larger bands between single/double digest. Faint bands would seem to be visible, however.
After further incubation, no additional bands appeared.

Next, the digests were done using two different aliquots of FastDigest buffer in parallel. After 25 minutes of incubation with enzymes, gel electrophoresis was conducted and the results were as follows;

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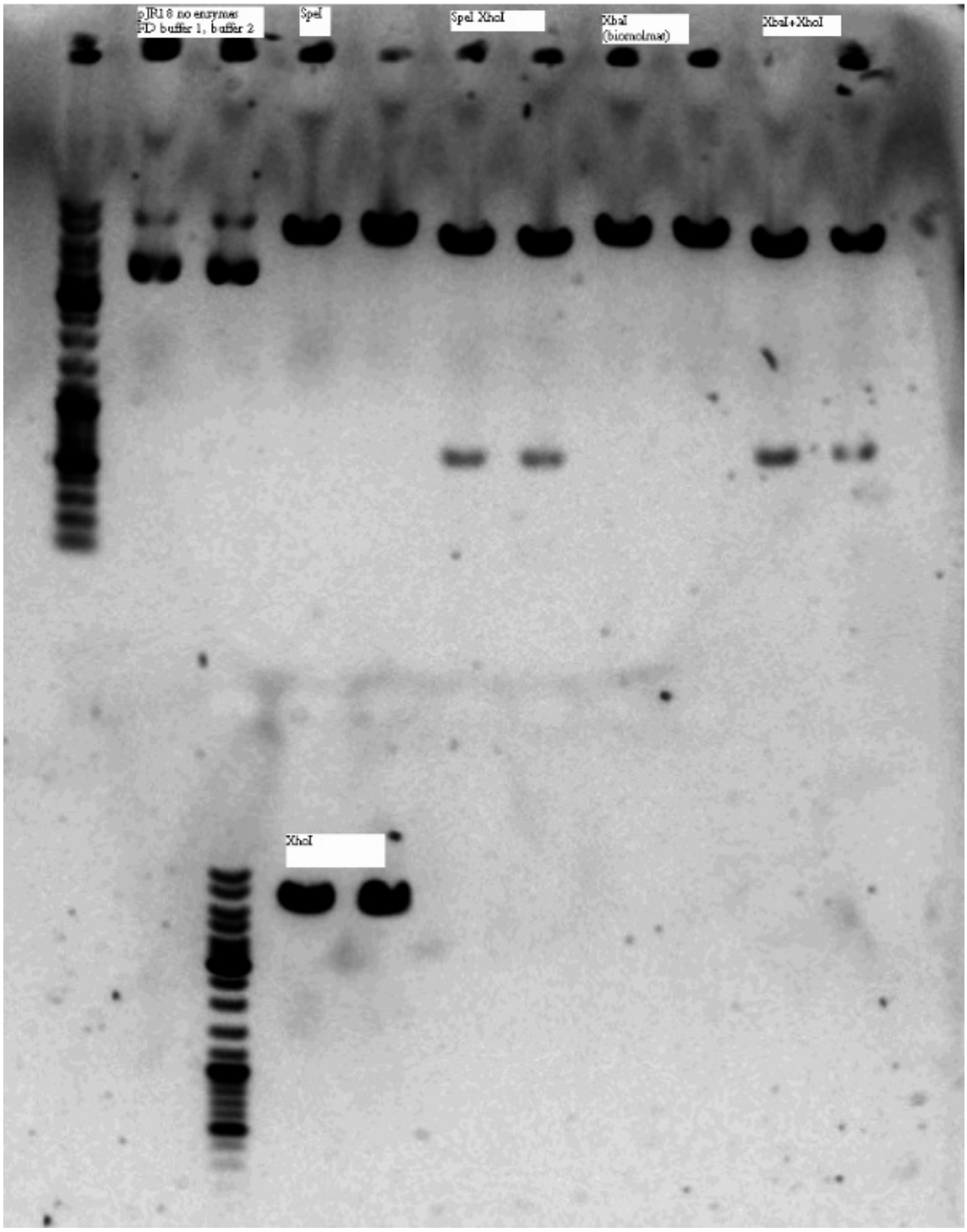


(from left; uncut enzyme with buffer aliquots 1 and 2, SpeI with same aliquots, SpeI+XhoI, XbaI(biomomat), XbaI+XhoI, XhoI)

As no more incorrect bands were observed, it would seem that the problem causing incorrect bands was with the Fast Digest buffer aliquot; there are many aliquots of buffer in the biomolecular materials common stock (where the buffer was used from), and it could be that one of them contains contamination, and this one was used in the digests that showed an additional band. Further testing with all the aliquots would be needed to confirm this, but the two aliquots tested last were marked (I and II on the caps), and they appear to be fine.

As it seems that there is nothing wrong with the template, however, it was decided to not continue the attempts to PCR-linearize the template. Although it's possible that there was some correclty linearized backbone from the earliest PCRs, these were done with the pRS415-GAL template which might cause some future problems. Further troubleshooting would likely not clarify the mystery of why the PCR worked earlier (maybe someone did something wrong which luckily caused it work a couple of times but not afterwards), so it was decided to continue the stress promoter construction with yeast recombination cloning instead of gibson assembly.

06-29/07-01 pJR17, pJR18, pUG6 Plasmid purification and concentration

Project: Promoter
Authors: Pihla Savola
Dates: 2016-06-29 to 2016-07-01

WEDNESDAY, 6/29

Liquid cultures of pJR17, pJR18 and pUG6 in TOP10 were prepared in LB+ampicillin (5ml + 5 ul) and grown overnight at 37 C with shaking.

THURSDAY, 6/30

Plasmid purification and concentration. MiniPrep of liquid cultures was done with Macherey-Nagel NucleoSpin plasmid miniprep kit and protocol. There were four preps of each plasmid, concentrations measured with nanodrop are below:

Table1

	A	B
1	Plasmid	Concentration (ng/ul)
2	pJR17	49.4
3		53.8
4		54.1
5		54.3
6		
7	pJR18	44.5
8		50.3
9		51
10		51.9
11		
12	pUG6	28.7
13		29.2
14		32.2
15		35.8

15 mL liquid cultures LB+ampicillin were prepared of pJR17 and pJR18 plasmids in LB+ampicillin to get higher plasmid concentrations.

FRIDAY, 7/1

MiniPrep of plasmidss. Cultures were distributed so that the 15 mL culture volume was eluted into two solutions of 50 uL. Protocol from NuceloSpin Plasmid Macherey-Nagel was used. Plasmid concentrations were determined by Nanodrop.

Table2

	A	B
1	Plasmid	Concentration (ng/ul)
2	pJR17	616.5
3	pJR17	537.6
4		
5	pJR18	507.5
6	pJR18	448

06-29/07-19 Venus YFP cloning, amplification and expression (for positive control)

Project: Promoter
Authors: Hele Haapaniemi
Dates: 2016-06-29 to 2016-08-18

WEDNESDAY, 6/29

This entry describes the cloning of Venus YFP to pRS415 plasmids with GPD promoter (pJR17 = pRS415 with a 700 bp insert) and GAL promoter (pJR18 = pRS415 with a 700 bp insert). Here's also details of the verification and expression + setup testing of Venus.

Venus YFP gBlock was resuspended according to the gBlock suspension protocol.

TUESDAY, 7/5

Venus gBlock was amplified using following mix for PCR:

Table9

	A	B
1		20ul reaction
2	nuclease free water	adjust to 20 ul
3	5xPhusion HF buffer	4
4	10mM dNTPs	0.4
5	10 uM forward primer	1
6	10 uM reverse primer	1
7	gBlock (1 ng/ug)	1
8	Phusion polymerase	0.2

Control was made without template. Two parallel samples were prepaired.
10 uM dilutions from primers and gBlock were made the previous day (and stored in freezer).

Following PCR conditions were used:

Table10

	A	B	C	D
1	step	cycles	temp	time
2	initial denaturation	1	98	30s
3	denaturation	5	98	10s
4	annealing	5	55	20s
5	extension	5	72	15s
6	denaturation	10	98	10s
7	annealing	10	59	20s
8	extension	10	72	15s
9	final extension	1	72	5min
10	hold	1	4	hold
11				

PCR products were run on gel and purified using PCR purification kit.

Venus gBLOCK.tif

Final concentration of PCR product was measured with nanodrop and it was 15.3 ng/uL. This was too little concentration for restriction digest of pJR17 and pJR18 +Venus so PCR will be done again on next day.

WEDNESDAY, 7/6

PCR amplification of gBLOCK venus was conducted again with same PCR mix as yeseterday (Table9) except amount of dNTP was 1 uL instead of 0.4 uL. (HUOM!! dNTPs should be 0,4 ul.). PCR was carried out with same conditions as yesterday (table 10) except cycles of last cycle step was increased to 20.

PCR products were run on 0.7% agarose gel (loadng order: ruler, negative control, venus, venus).

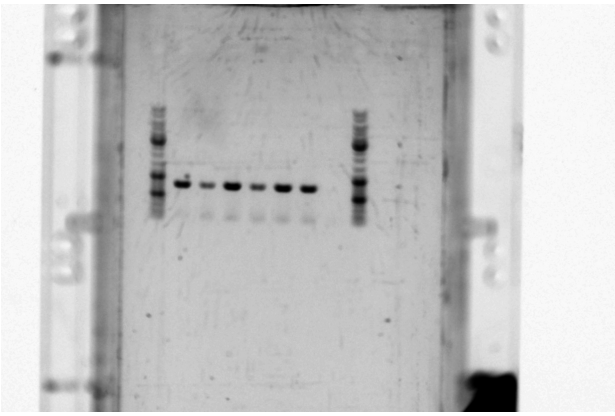
Venus Block 6.7.JPG

PCR products were purified with PCR purification kit. Final concentrations of PCR products was measured with Nanodrop. We got concentrations of 33 ng/ul and 38 ng/ul.

THURSDAY, 7/7

PCR of venus gBLOCK was conducted again with same conditions as yesterday and run on 0.7% agarose gel. (loading order ruler, venusX6, NC).

venus gBlock 7.7.tif



In PCR product purification was done with PCR purification kit but products were combined into one tube before beginning. Final concentrations were measured with nanodrop and they were 84 ng/uL and 150 ng/uL.

Restriction digest was conducted for PCR-amplified VenusYFP gBlocks and pJR018 and pJR018 plasmids. For restriction digests, the following materials were combined;

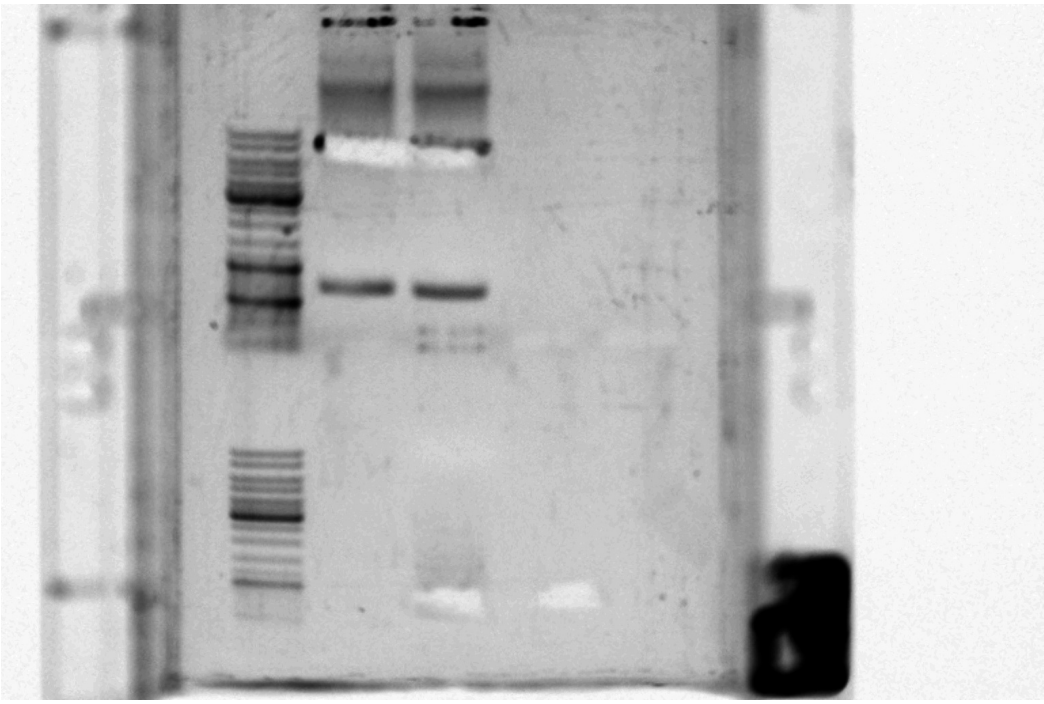
Table1

	A	B	C	D
1		Venus (150ng/uL)	pJR017 (606ng/uL?)	pJR018 (501ng/uL)
2	H2O	18 uL	28,7 uL	28 uL
3	FD Buffer 10X	4 uL	4 uL	4 uL
4	FD BcuI (SpeI)	2 uL	2 uL	2 uL
5	XhoI	2 uL (FD XhoI)	2 uL (NEB XhoI)	2 uL (NEB XhoI)
6	Template DNA	13 uL	3,3 uL	4 uL
7	Total volume	40 uL	40 uL	40 uL

Restriction reactions were incubated at 37 C for 1 hour (for complete digestion of NEB XhoI). Heat-inactivation of restriction enzymes was conducted at 80 C for 20 minutes.

The restriction digest was run on a gel (0,7% agarose) and the correct restriction products (pJR017+pJR018 around 6800 bp, Venus around 700 bp) were verified and cut out of the gel. Gel purification was conducted using the NuclepSpin Gel and PCR cleanup kit and attached protocol (Macherey-Nagel), and the concentrations of the purified fragments was measured.

pJR17,18, VENUS RESTRICTION CUT ALL.img.tif



Ligation was conducted according to the attached protocol for Venus+pJR018 and Venus+pJR017 restriction product. Concentraions of restriction digest products were measured with nanodrop and concentraions were Venus: 37.5 ng/uL, pJR17 33.6 ng/uL and pJR18 27.6 ng/uL).

Table2

	A	B	C
1		pJR18+Venus	pJR17+Venus
2	Vector DNA	1.81 uL	1.48 uL
3	Insert DNA	0.42 uL	0.42 uL
4	10 X Ligase buffer	2 uL	2 uL
5	T4 DNA ligase	1 uL	1 uL
6	H20	14.77 uL	15.1 uL
7		20 uL	20 uL

Ligation reaction was incubated at room temperature for 10 minutes and then 10 minutes at 65 C to inactivate the ligase.

5 uL of the ligation reaction was transformed, according to attached protocol, to TOP10 competent cells.
No colonies were obtained on any of the plates; failure of the ligation could be due to using 10X ligase buffer from NEB and DNA ligase from ThermoFisher. Additionally, an extended ligation time could be beneficial. These changes will be made in a second ligation attempt.

MONDAY, 7/11

Ligation of pJR17 and pJR18 with venus was done again with attached protocol (due to the last time failure). Following mixes were made

Table3

	A	B	C
1		pJR17+Venus	pJR18+venus
2	Vector DNA	1.81 ul (33.6 ng/uL)	1.48 uL (27.6 ng/uL)
3	Insert (venus) DNA	0.42 uL (37.5 ng/uL)	0.42 uL (37.5 ng/uL)
4	10X T4 DNA ligase buffer (thermo fisher)	2 uL	2 uL
5	T4 DNA ligase	1 uL	1 uL
6	H20	14.77 uL	15.1 uL
7	Total volume	20 uL	20 uL

Ligation reaction was incubated at roomtemperature for 2h. Ligation reaction was inactivated with heat block at 65 C for 10 min

Ligated reactions were transformed to TOP10 comptent cells according attached protocol. Only exception was that bacterteria/DNA mixture was incubated for 45 min instead of 30 min at 37 C with shaking. Plated volumes on antibiotic plates were for both ligations 50 uL (X2), 100 uL and 150 uL. Plates were left to incubate for O/N at 37 C

TUESDAY, 7/12

Every plate contained colonies.
Colony PCR was made from the plates with following mastermix

Table4

	A	B
1	10X dreamtaq buffer	13 uL
2	dNTP (2 mM)	13 uL
3	FW primer (EKO025)	2.6 uL
4	RW primer (EK028)	2.6 uL
5	Dreamtaq polymerase (added last)	1.3 uL
6	H20	97.5 uL

From mastermix 11 10 uL samples were taken (negative control, positive control and 9 normal samples). To normal samples colonies from pJR18 plate was inoculated and to negative control colonie from MlrA plate and for positive control pJR18 plasmid. PCR was run on following conditions

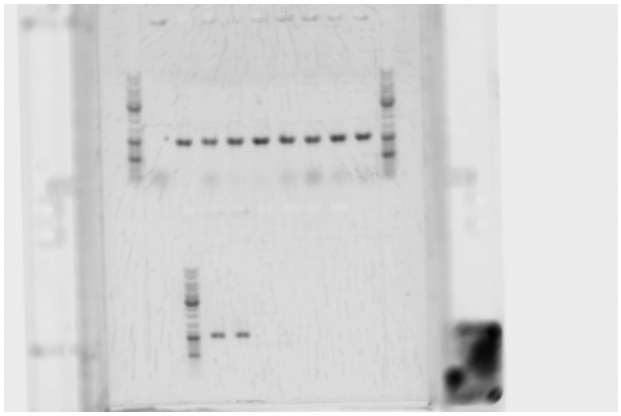
Table5

	A	B	C	D
1	Step	T (C)	time	Cycle(s)
2	Step	95	3 min	1
3	Denaturation	95	30 s	30
4	Annealing	50	30 s	30
5	Extension	72	1 min 10 s	30
6	Final extension	72	15 min	1
7		4	∞	

WEDNESDAY, 7/13

From yesterdays PCR products (pJR18+venus) were run on 0.7% agarose gel (loading order first row: ruler NC, PC, 1 ,2 ,3 ,4 ,5 6, 7 ruler second row: ruler 8, 9). Gel was imaged and every sample except NC were on 1kb mark.

Venus colony pcr pJR18.tif



For Colony PCR of pJR17+ Venus following mastermix was prepared

Table6

	A	B
1	10X dreamtaq buffer	8 uL
2	dNTP (2 mM)	8 uL
3	FW primer (EKO026)	1.6 uL (0.2 uM)
4	RW primer (EK028)	1.6 uL (0.2 uM)
5	Dreamtaq polymerase (added last)	0.8 uL
6	H20	60 uL
7	Total volume	80 uL

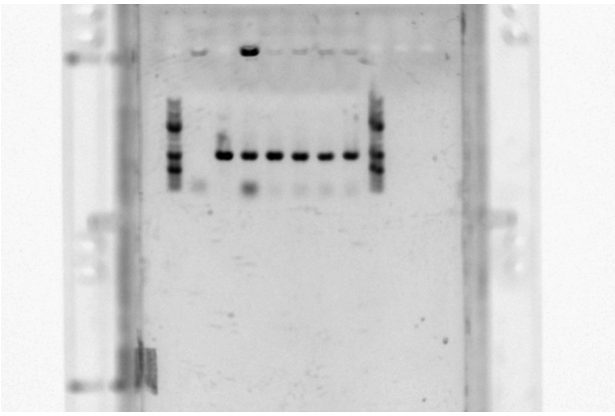
From mastermix 7 pcr samples were prepared (negative control, positive control and 5 colony samples from pJR17+ venus plate). To negative control colony from MlrA plate was inoculate, to positive control plasmid pJR17 was put and to colony samples colonies from pJR17+ venus plates was inoculated. PCR was run with following conditions.

Table7

	A	B	C	D
1	Step	T (C)	time	Cycle(s)
2	Step	95	3 min	1
3	Denaturation	95	30 s	30
4	Annealing	50	30 s	30
5	Extension	72	1 min 10 s	30
6	Final extension	72	15 min	1
7		4	↔	

PCR products were run on 0.7% agarose gel and gel was imaged and all bands except negative control were on right spot in other words in 1kb mark (loading order: ruler, NC, PC, 1, 2, 3, 4 ,5, ruler)

pJR17 colony PCR.tif



6 Liquid cultures were prepared on pJR17+venus (3 samples; colonies 3,4,5) and pJR18+venus (3 samples; colonies 4,7,10. Colonies were inoculated into 5 ml LB and 5 uL ampicil. Cultures were incubated O/N in shaking at 37 C.

THURSDAY, 7/14

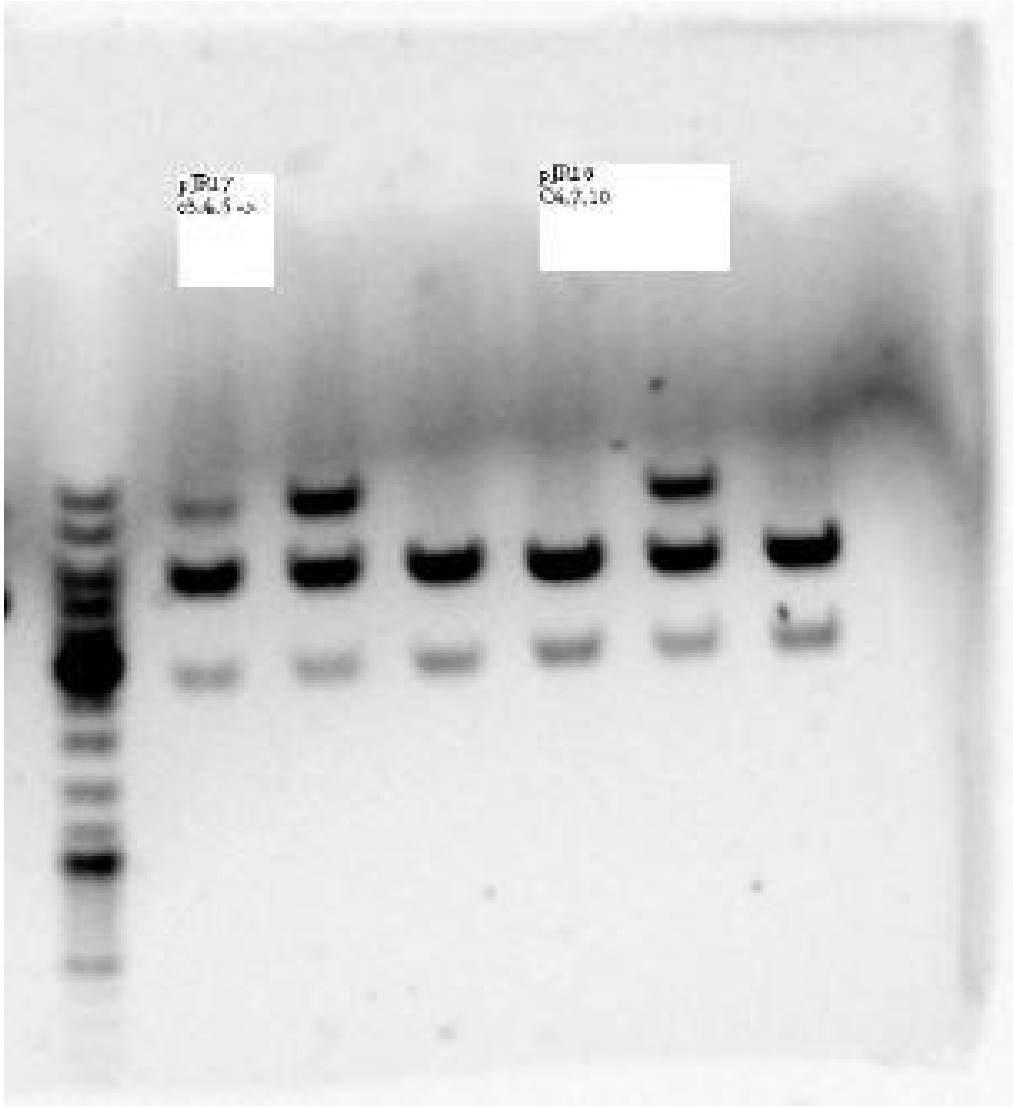
Miniprep of O/N liquid cultures of pJR17+Venus and pJR18+Venus (6 total, 3 for each) was done with attached protocol (with Macherey-nagel DNA, RNA and protein purification kit). Final plasmid concentrations were measured with minidrop and the concentrations were: pJR17 C-3: 246.6 ng/uL, pJR17 C-4: 275.7 ng/uL, pJR17 C-5: 278,9 ng/uL, pJR18 C-4:283.4 ng/uL, pJR18 C-7:249,1 ng/uL pJR18 C-10: 376.3 ng/uL.
In order to make sure that our Venus constructs are right ones, restriction digest was carried out with EcORI HF and Nde 1 enzymes. With these enzymes the products should be kb and kb. Following solutions were prepared for restriction digest.

Table8

	A	B	C	D	E	F	G
1		pJR17 C3	pJR17 C4	pJR17 C5	pJR18 C4	pJR18 C7	pJR18 C10
2	10X cut smart buffer	2 ul		2 ul	2 ul	2 ul	2 ul
3	Eco RI HF	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
4	Nde 1	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul	0.5 ul
5	Template DNA (venus construct)	2.03 ul	1.81 uL	1.79 uL	1.76 uL	2.00 uL	1.33 uL
6	H20	14.97 ul	15.19 uL	15.21 uL	15.24 uL	15.00 uL	15.67 uL
7	Total volume	20 uL	20 uL	20 uL	20 uL	20 uL	20 uL

Restriction digest products were verified with 0.7% agarose gel. The first gel did not succeed for unknown reason (ruler was not readable), but the next one showed that our restriction products were right size (loading order ruler, pJR17: C3, C4,C5, pJR18: C4, C7, C10).

pJR17 andpJR18 Venus restriction cut.JPG



MONDAY, 7/18

Liquid culture of W303A (s.cerevisiae) was prepared into 5 ml YPD media. Cultures were left on O\N incubation in shaking at 30 C.

TUESDAY, 7/19

Competent cells were prepared as followingly: From O\N W303A cultures new liquid cultures were prepaired so that starting OD600 was 0.5 by diluting with YPD. Cultures were growth at 30C in shaking until the OD600 was between 1.5-2.0 (we got 1.5). After this tubes were fuged and pellets were washed with 30 ml sterile H20. Pellets were washed with 500 uL of solution A (in 10 ml: 1 ml 10X TE, 1 mL 1M LiAc, 8 ml ddH2O) and resuspended with solution A with final volumes of 300 uL and 200 uL.

Plasmids containing pJR17+Venus(C-3) and pJR18+Venus(C-10) were transformed with attached protocol (Competent yeast cells+LiAc transformations). Transformation solutions were plated on minimal media (minus leucine) and left to grow on 30 C, for 2 days.

MONDAY, 7/25

Liquid cultures of pJR17+venus and pJR18+venus was prepared. Cells were inoculated into 5ml SD-media (-leucine+2 glucose) and negative control was prepared into SD-media (+all amino acids + 2 glucose) containing all plasmids with same strain yeast which did not contain plasmid. Cultures were incubated in shaking O/N at 30 C.

Sequencing samples were prepared of pJR17+Venus(C-3) and pJR18+Venus(C-10). (Eurofins valueread). Premixed samples were prepared by combining 15 uL of template dna (50-100 ng/uL) and 2 uL of 10 uM primers: The following samples (template+primer) were prepared:

- pJR17+Venus(C-3) + EK26
- pJR18+Venus(C-10) + EK25

TUESDAY, 7/26

Absorbance (524nm) and absorbance spectrum was measured from O/N culture of pJR17+Venus. No difference was observed between sample and negative control.

Gal induction was conduted from O/N pJR18+Venus in 5ml media (1ml culture+ 4ml induction media (minimal media -leucine+ 2 %gal)). Cells were incubated O/N in shaking at 30 C. Negative control was done from same O/N culture but 2% raffinose was used instead of galactose.

WEDNESDAY, 7/27

Absorbance (514 nm) of pJR18+venus gal induced O/N culture was measured. No difference was observed between sample and negative control (514: pJR18 2.621, NC: 2.262, OD600 pJR18:2,523, NC: 2,169).

It was realized that these absorbance measurements are not enough for fluoresence detection and thus, next time fluorensce will be measured with fluorometer of plate reader.

MONDAY, 8/1

Liquid cultures of pJR17/18+Venus were prepared. Cells were inoculated into 5ml SD-media (-leucine+2% glucose). Negative control was prepared with normal w303A without plasmid into 5ml SD-media (+all amino acids + 2 %glucose). Cells were left to grow at 30 C in shaking O/N

TUESDAY, 8/2

Sequencing results for Venus constructs (pAH04-C3 and pAH05-C10) verified the correct sequences.

Absorbance (OD600) and fluoresence was measured from pJR17+Venus O/N culture with plate reader. First the OD of samples and controls were balanced (OD distillations, NC: 0,4. pJR17+venus: 0.4 and 0.56). Absorbance was measured with 600nm wavelength and fluoresence with 502 excication and 528nm emission. (NOTE: with Venus YFP the higest fluorescnece excication is on 515 nm but the plate reader could not read it and 502-528 was the smallest difference that it could read properly). Fluoresence values are listed below

Table12

	A	B	C
1	Sample	Fluoresence	Fluoresnce blanked
2	Blank	8904	0
3	Negatice control	8117.5	-786.5
4	Sample pJR17+Venus OD=0,4	10498.5	1594.5
5	Sample pJR17+venus OD= 0,5	10465.5	1561.5

It can be seen that our constructs produced some fluoresence. However, it can be also seen that negative control (Cells no plasmid for YFP production) has lower fluoresnce value than blank (Fresh SD-media where cells grow). This maybe due reason that some compounds in our SD-media are fluoresent in range of YFP. All data is attached below in excel.

PJR17+VENUS Fluoresence measurements.xlsx

pJR18+Venus O/N culture was refreshed to 5 mL of fresh SD-media (-leucine+ 2% raffinose) for 4 h. First the cells were centrifuged down (5min, 3000 g), the supernatant discarderd, and washed with same media (2% raffinose) and then media was changed. After refreshing pJR18+Venus culture was induced with galactose. First the 5ml culture was split to 2.8ml and 2.2ml samples where another is the sample and another serves as negative control (without induction). 0.131mL 40% galactose was added to sample and 0.262ml 20% raffinose was added to negative control (final concentration of raffinose and galactose 2%). Induced cultures were left to incubate on 30C O/N in shaking.

WEDNESDAY, 8/3

Absorbance and fluoresence was measured from galactose induced pJR18+Venus construct with plate reader. Absorbance (OD600) was measured with 600nm wavelenght and fluoresence with 502nm excication and 528nm emission. Following dilutions with following ODs were prepaired before measurements: NC OD= 0.9. pJR18+venus: OD= 0.97. Fluoresence was also measured from undiluted samples.

Table13

	A	B	C
1	Sample	Fluoresence value	Fluoresence blank read
2	Blank	13786	0
3	Negative control (no dilution	7568	-6133
4	Negative control (OD=0.9)	10622	-3079
5	Sample (No diluted)	21350	7649
6	Sample (OD=0.97)	18919	5218

It can be seen again that our constructs have produced some fluoresence but negative control (media with cells) has lower fluoresence than blank (fresh media without cells). All data is attached below in excel file.

pJR18+Venus Fluoresence measurements.xlsx

TUESDAY, 8/16

In fluoresence measurements of YFP, it was observed that negative control samples had smaller fluoresence values than blank. Due to this it was decided to measure fluoresence spectrum of "used media" (media that has been used to grow yeast) and fresh media in order to see if our media (SD -leu +2% sugar either glu, gal or raf) has high base fluoresence level.

Liquid culture of pJR18+Venus was prepared. Cells were inoculated into 1ml of SD-media (-leu + 2 % glu) and cells were left to grow O/N in 30 C in shaking.

WEDNESDAY, 8/17

O/N culture was moved into 1.5ml eppendorf and cells were spinned down (3600 rpm, 3min) and 100 ul of suprenatant was pipeted into 96 well plate. This will serve as "used media" sample. 100 uL of fresh no used SD-media was also pipeted into 96 well plate and fluoresence and fluoresence spectrum was measured with plate reader. (Plate order A1: Fresh media, B1: used media). Fluoresence measurements are in table below and all measure data is attached in excel file below.

Table14

	A	B
1	Sample	Fluoresence
2	Fresh media	37012
3	Used media	31345

Fresh media and used media fluoresence data.xlsx

It was observed that used media had smaller fluoresence than fresh media.

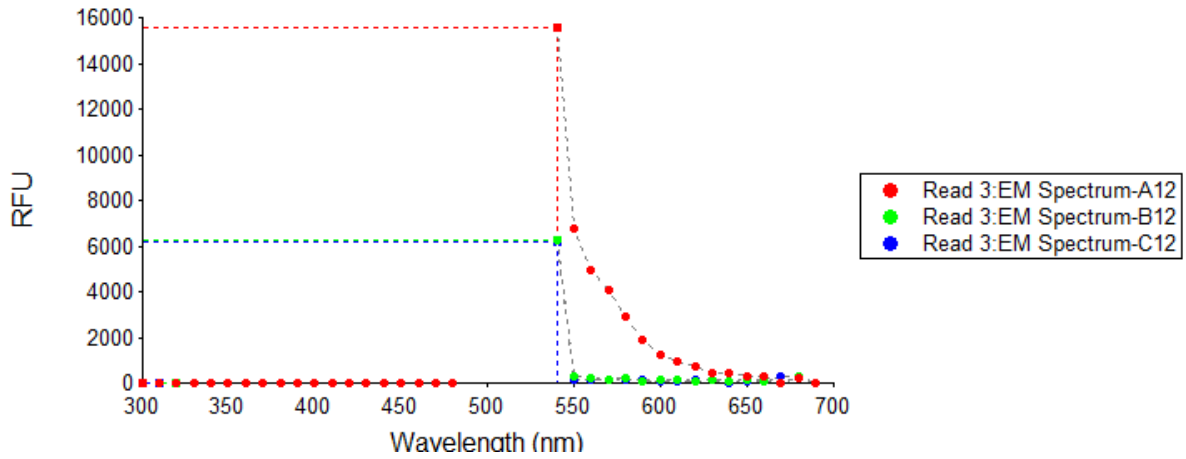
THURSDAY, 8/18

Because it appears that SD-media has fluoresence in same area than Venus-YFP it was decided to see if it has any additional excication/emission spikes which could be measured instead of the main spike.

1ml liquid cultures of pJR17+Venus was prepared. Cells were inoculated into SD-media (-leu +2% glucose) and cells were left to grown at 30 C in shaking for 5h. After 5h fluoresence spectrum was measured with plate reader. Before flurosence measurements OD600 was measured and it was 0.4. 1 ml culture was divided into 2 1.5ml eppendorf tubes. In another tube cells were spinned down (3600 rpm, 3min) and 100 ul of media was piped into 96 well plate. Also 100 ul of cell culture and fresh media was pipted into 96 well plate. Fluorense spectrum was measured with plate reader. (Plate order A12: culture with cells, B12: used media, C12: fresh media).

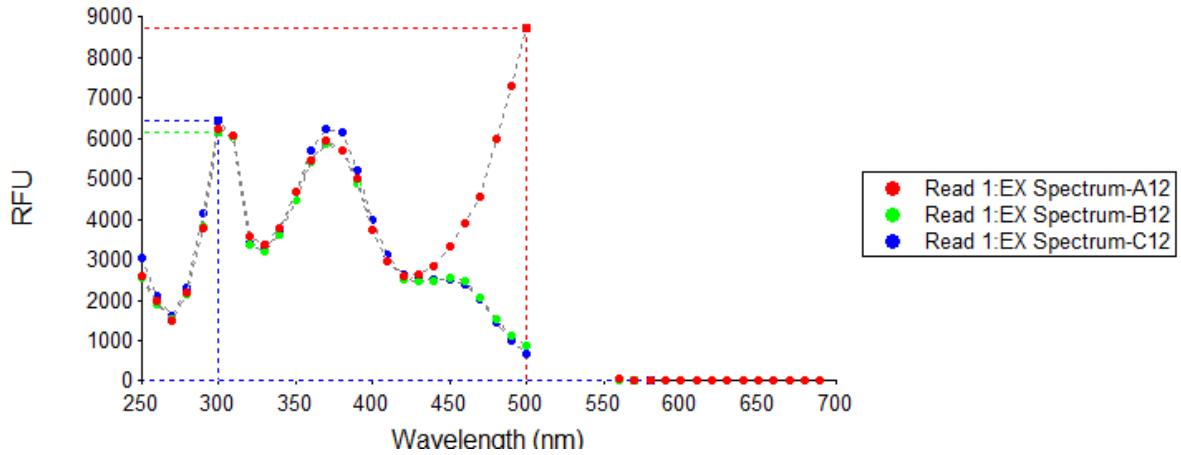
It was concluded that no other emission spike exists. Data in excel and pictures of emission and excication spectrums are attached below. NOTE: it must be taken into account that values between wavelenghts 480-550nm in both excitation and emission measurements were too high for plate reader to read. That's why there is a gap in emission/excitation figures.

pjr17+venus emission spectrum.png



A12=Culture with cells, B12= used media, C12=fresh media

pjr17+venus excitation curve.png



16.8.16 pjr17+venus spectrum.xlsx

No additional emission spike was observed.

07-06 Verifying pJR18 template

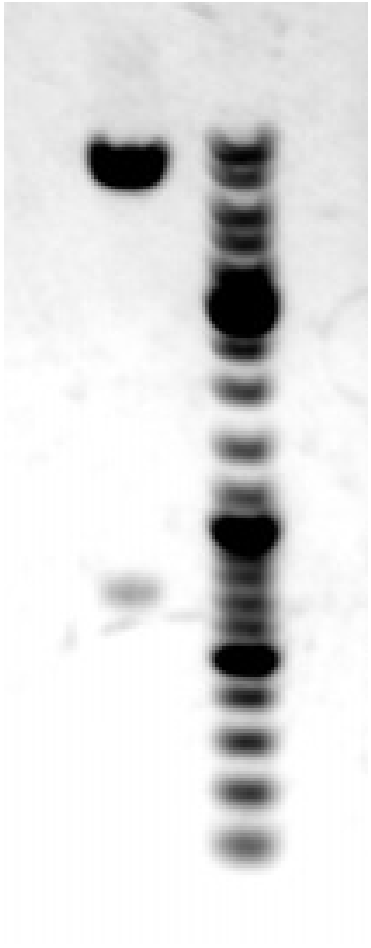
Project: Promoter
Authors: Hele Haapaniemi
Date: 2016-07-06

WEDNESDAY, 7/6

GeneArt has been unable to get colonies using the pRS415-GAL template for cloning of synthesized genes (MlrAY, MlrAYAlpha, MlrAY3Alpha) with SpeI and XhoI restriction sites, reporting extra bands when cutting the template even when using different enzyme aliquots and coming to the conclusion that something is wrong with the template. For this reason, work with pRS415-GAL template was discontinued, as there might be something unknown wrong with it, and instead a switch was done to using pJR018 for cloning and PCR purposes where pRS415-GAL had been used; pJR018 is the same base plasmid as pRS415-GAL, but contains a 678 bp insert between SpeI and XhoI restriction sites.

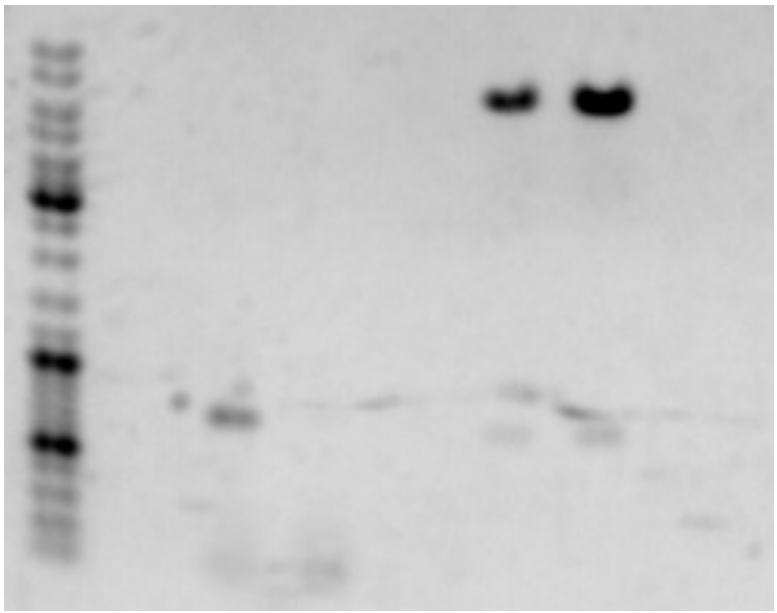
To verify pJR18 before sending it for cloning at GeneArt, and using it for PCR for stress promoter assembly, diagnostic restriction digest was conducted according to the attached protocol for pJR18 template.
Digest was run on gel, and the following image was obtained.

clipboard_2016-07-20_14:26:26.png



As the larger band appears to be larger than expected, the digest was rerun;

clipboard_2016-07-20_14:29:24.png



(the two samples on the right are the pJR18 template restriction digest)

Based on this picture, the bands would appear to be in the correct size (700 bp, and the larger band 6.7 kb). No extra bands are observed, so the template was concluded to be okay for use.

07-19/20 pJR018 plasmid amplification + check

Project: Promoter
Authors: Hele Haapaniemi
Dates: 2016-07-19 to 2016-07-20

TUESDAY, 7/19

Liquid culture of 20 ml LB with ampicillin was incolulated from a fresh plate containing TOP10 E. coli with pJR018. The cells were grown overnight at 37 C with shaking.

WEDNESDAY, 7/20

pJR018 plasmids was isolated from the culture using the NucleoSpin Plasmid miniprep kit (Macherey-Nagel), and the attached protocol. The miniprep was done into 4 separate 1.5 ml eppendorf tubes, and the plasmid concentration in each tube was measured with Nanodrop. Concentrations can be found in Table 1. The tubes were labeled with pJR18, the date, and numbers 1-4.

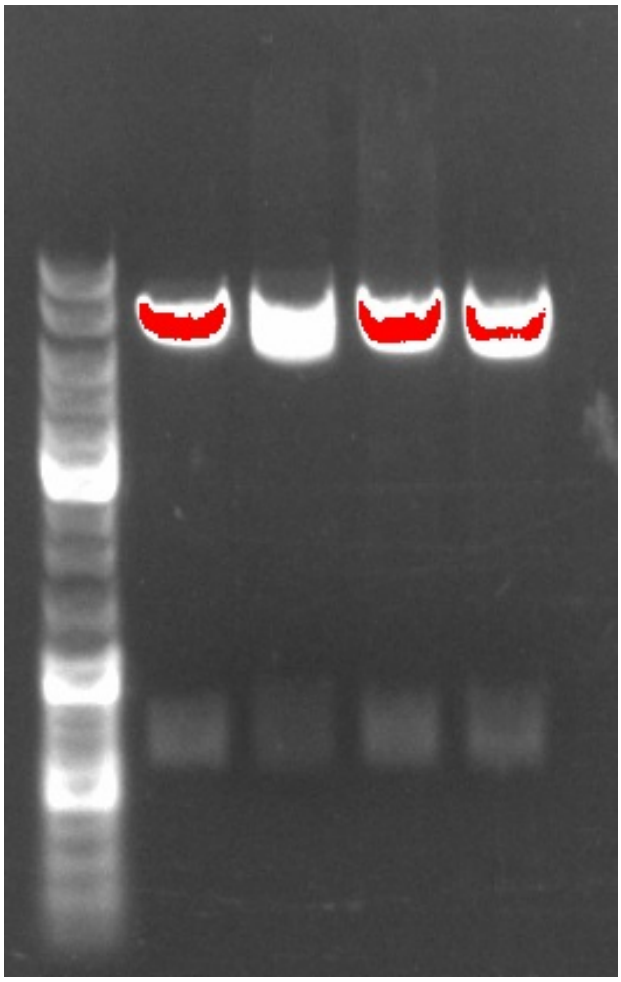
Table1

	A	B
1	Tube labeled	Concentration (ng/uL)
2	pJR18 20/7 1	707.2
3	pJR18 20/7 2	805.8
4	pJR18 20/7 3	688
5	pJR18 20/7 4	696.4

To verify that there was no problem with the template, diagnostic restriction digest was conducted using the attached protocol and the enzymes BcuI and XhoI. As a deviation from the protocol, 1 uL of each template was used, and therefore larger template amounts than 500 ng.

To visualize the results of the digest, agarose gel electrophoresis was conducted. Gel electrophoresis was done using Ethidium bromide instead of SyBr Safe, which had been used in all earlier electrophoresis runs.

2016-07-20 pJR18check.tif



(From left; pJR18 1, pJR18 2, pJR18 3, pJR18 4)

The restriction digest appears to produce the correct bands and no additional products.

07-19/25 Yeast Recombination for Stress Promoter Assembly

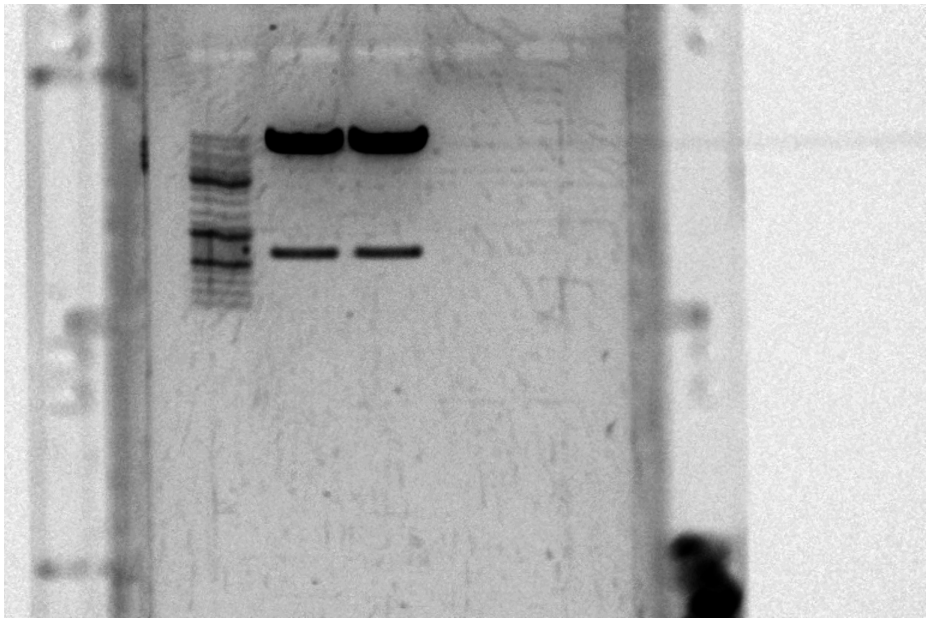
Project: Promoter
Authors: Hele Haapaniemi
Dates: 2016-07-19 to 2016-08-26

TUESDAY, 7/19

Linearization of pJR018 for recombination was conducted by restriction digest according to the attached protocol. For the pJR018 template, 9 uL of 448 ng/uL and 4.5 uL of 507 ng/uL solutions were used for a total of 6.3 ug of template DNA in a 13.5 uL volume.

After 1 hour of incubation at 37 C, the restriction digest was run on a 0.7 % agarose gel, using purple loading dye.

pJR18restriction for yeast recombination.tif



(The pJR018 restriction, divided into two wells.)

The linearized plasmid band was cut out from the gel and purified using the GeneJet Gel extraction kit (ThermoFisher). Concentrations of the purified products (purified in 2 separate tubes) were measured with nanodrop; concentrations of 22.6 ng/uL and 26.5 uL were measured (eluted in 50 uL).

The linearized, purified plasmid backbones fragments were transformed into yeast along with the insert (gBlock containing stress promoter sequence followed by Venus YFP. Inserts were flanked on both sides by 30 bp homology to the sites where they should be recombined with the plasmid.

For the transformation of each construct, the following amounts of DNA were used;

Table1

	A	B	C	D
1		CTT1_Venus	CCP1_Venus	TSA1_Venus
2	pJR18 backbone (22.6 ng/uL solution)	-	34.5 uL	18 uL
3	pJR18 backbone (26.5 ng/uL solution)	29.5 uL	-	15 uL
4	pJR18 backbone in total	779.1 ng	770.66 ng	816 ng
5	Insert (10 ng/uL solution)	10 uL (100 ng)	10 uL (100 ng)	10 uL (100 ng)

The transformation mixtures were plated on minimal media plates without leucine and grown in 30 C. After two days, visible colonies were observed, but incubation was continued over the weekend; plates were kept in plastic bags during incubation to help prevent drying.

MONDAY, 7/25

Incubated plates were collected and DNA preparation was conducted according to the attached protocol for each plate to obtain DNA, including the hopefully recombined plasmids, from the cells. DNA was suspended in 50 uL of water.
5 uL of prepared DNA of TSA1, CTT1 and CCP1 were transformed to competent TOP10 cells according to bacterial transformation protocol. Different quantities (50/100/150ul) were plated and grown overnight in 37°C.

TUESDAY, 7/26

No colonies were observed from the plates.

Left-overs from previous days's transformation were plated.

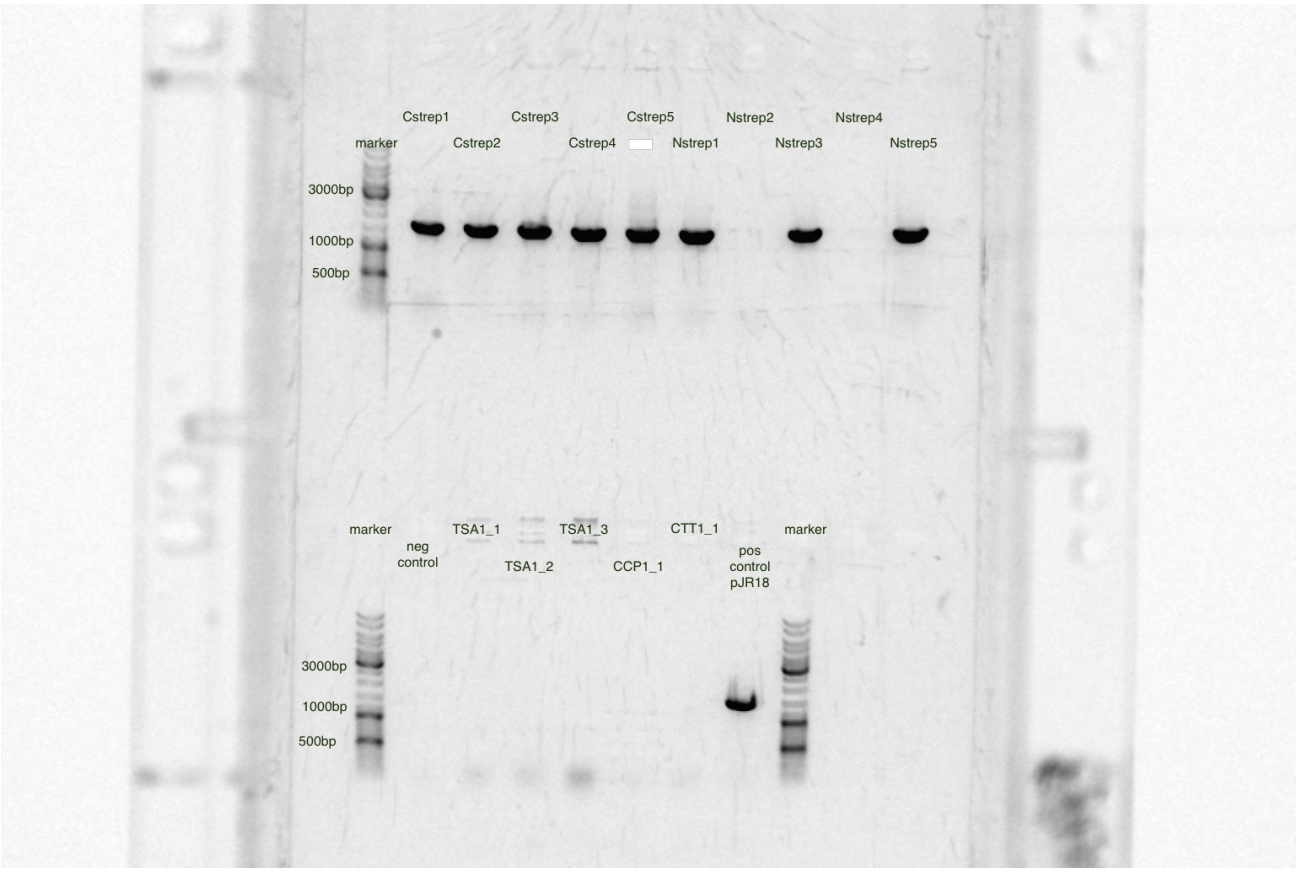
New transformation according to the same protocol was performed and this time 200ul was plated.

WEDNESDAY, 7/27

Very few colonies appeared after O/N incubation.

Inserts were analysed with colony PCR according the colony PCR protocol.

NCstrep+promoter colony PCR 27.7.16.tif



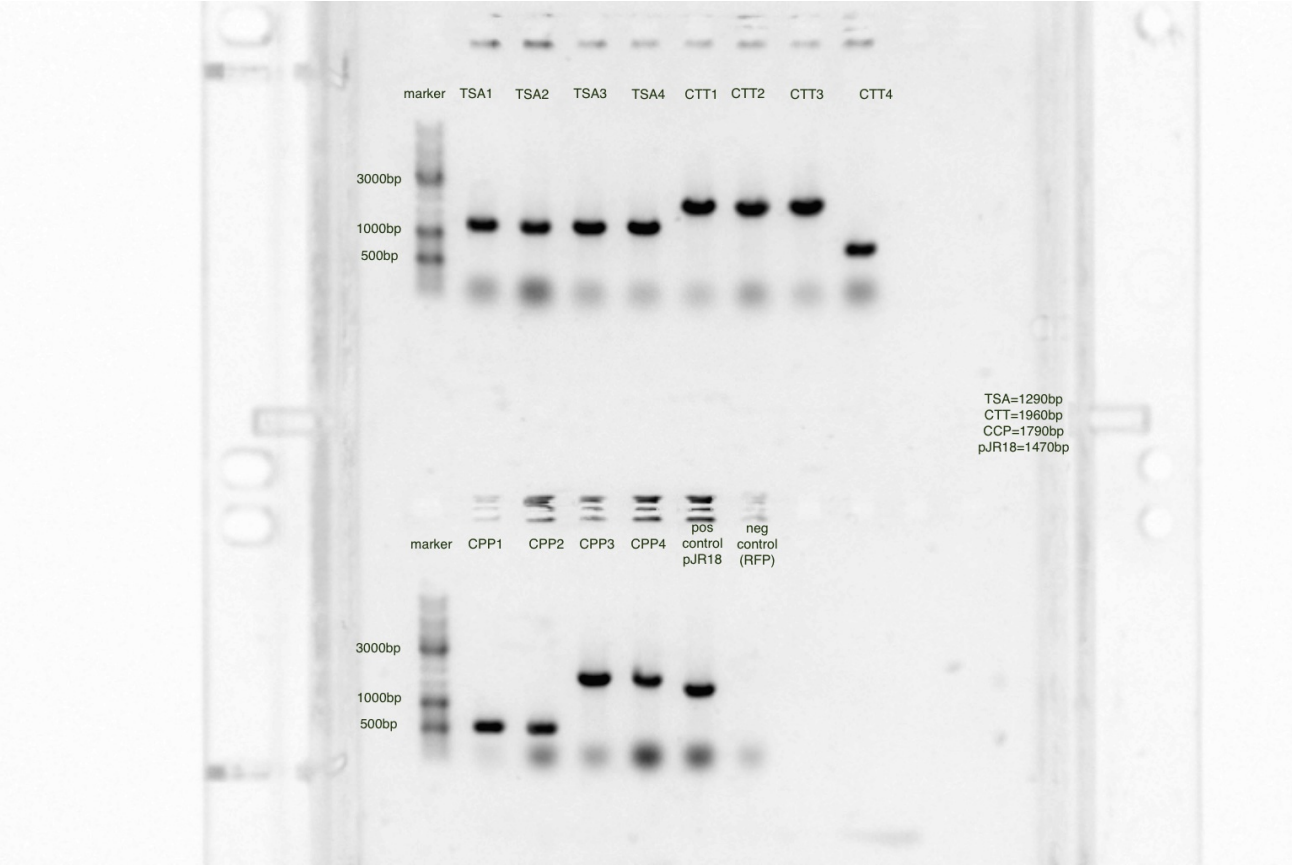
No bands could be seen.

Transformation of the three constructs purified from yeast was tried using electroporation according to the attached protocol. 2 uL of the DNA purified from yeast was used in the transformation. Amounts of 20 uL and 200 uL were plated on LB-Amp plates from each transformation.

THURSDAY, 7/28

Colonies were observed on all the plates transformed with electroporation. Colony PCR was conducted to analyze the colonies.

promoter colony PCR_28.7.16.tif



According the colony PCR results all analysed TSA colonies contained the right insert, as well as CTT colonies 1-3 and CCP colonies 3 and 4. Liquid culture was made from three of each promoter insert containing colonies.

FRIDAY, 7/29

Miniprep was made from liquid cultures. Concentration of 300ng/ul to 500ng/ul were achieved.

TUESDAY, 8/2

Samples from TSA colony 2 (pAH03-C2), CTT colony 1 (pAH01-C1), and CCP colony 3 (pAH02-C3) were sent for sequencing. (Eurofins, Valueread)

For each sequencing reaction, in 1.5 mL eppendorfs, 15 uL of template DNA (in concentration 50-100 ng/uL) was prepared. The following were sent like that, to be used with the free eurofins standard primers:

- pAH01-C1: FW primer T3, RV primer eGFP_36uni
- pAH02-C3: FW primer T3, RV primer eGFP_36uni
- pAH03-C2: FW primer T3, RV primer eGFP_36uni

WEDNESDAY, 8/3

Diagnostic restriction digest was conducted on the plasmid DNA sent for sequencing and used for transformation into yeast (pAH01-C1, pAH02-C3, pAH03-C2) to provide additional verification on the correct insert. The following reactions were set up:

Table2

	A	B	C	D
1		pAH01-C1	pAH02-C3	pAH03-C2
2	CutSmart Buffer	2 uL	2 uL	2 uL
3	EcoRI HF	0.5 uL	0.5 uL	0.5 uL
4	NdeI	0.5 uL	0.5 uL	0.5 uL
5	Template DNA	1 uL (template concentration ~500 ng/uL)	2 uL (template concentration 298 ng/uL)	1 uL (template concentration ~500 ng/uL)
6	H20	16 uL	15 uL	16 uL
7	total	20 uL	20 uL	20 uL

Samples were incubated for 1 hour at 37 C. Results of the digest were verified by running a 7.5 uL sample on a 0.7 % agarose gel with purple loading dye

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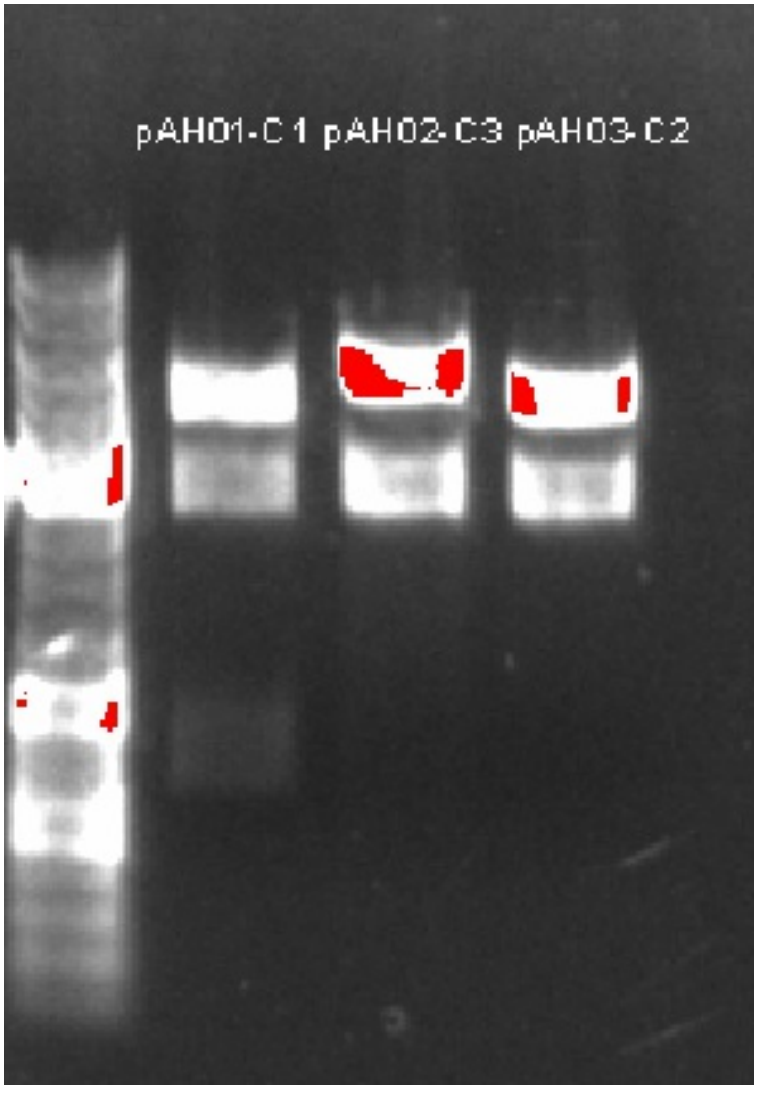

(from left; marker, pAH01-C1, pAH02-C3, pAH03-C2)

The gel seems to verify the correct inserts; without an insert, only EcoRI would cut to linearize the plasmid, but with the insert, multiple bands are produced.

For pAH01, bands of 4300, 2800 and 750 are expected; bands appear around these sizes; between 4000 and 5000, around 3000, and around 700 bp. For pAH02, bands of 4900 and 2800 are expected, and these are observed. For pAH02, bands of 4400 and 2800 are expected and observed,

TUESDAY, 8/9

Sequencing results revealed that pAH01-C1 was correct and will be used in future work, pAH02-C3 not and pAH03-C2 not. New colonies were sent: pAH02-C4 and pAH03-C3.

The promoter area in pAH02-C3 contained several point mutations, and that pAH03-C2 contained a single-base mutation in the promoter area as well as a deletion resulting in the abence of one amino acid near the C-terminus of the Venus-YFP. For this reason, different colonies were sent for sequencing and used in the following experiments.

TUESDAY, 8/16

pAH03-C3 is correct and will be used in future work. pAH02-C4 still remains unclear (short reads), so a new sample was sent for sequencing - with the same composition as before, but now with 1 % DMSO in the solution.

FRIDAY, 8/26

pAH02-C4 is not correct, but it will be used in the future all the same since it seems to work (See Fluorescence measurements of stress promoters and venus). pAH02-C4 contains two single base mutations in the promoter area. These mutations are before -300bp, so they don't affect the theoretical key transcription factor binding sites, but can nevertheless affect promoter function.

07-27 pJR18/pJR17-Venus amplification

Project: Promoter
Authors: Pihla Savola
Dates: 2016-07-25 to 2016-07-26

MONDAY, 7/25

Liquid culture was started from plates to amplify more pJR18-Venus-C10 and pJR17-Venus-C3. (These are the colonies that were used for transforming into yeast). Out of a LB+ampicillin culture grown for 7 hours, 1 ml was incoculated into 5 ml of fresh LB+ampicillin and grown overnight.

TUESDAY, 7/26

500 uL of the overnight culture was combined with 500 uL of 50% glycerol in a cryotube to make a glycerol stock, which were stored in -80 C.

The rest of the culture was processed to purify the plasmids using the NucleoSpin Plasmid miniprep kit and attached protocol (Macherey-Nagel). Concentrations were measured with Nanodrop (each was around 250 ng/uL) and purified plasmid solutions were stored in -20 C.

08-03/04 Yeast Transformation to W303a-leu

Project: Promoter
Authors: Hele Haapaniemi
Dates: 2016-08-02 to 2016-08-03

TUESDAY, 8/2

Competent yeast cells were prepared from W303-leu strain according the protocol and stored overnight at +4°C.

WEDNESDAY, 8/3

Transformation was done according the transformation protocol and reaction was plated on minimal media-leucine plates. A negative control from competent cells (plating on MM plates) as well as positive control (plating on YPD plates) were made.

Following plasmids were transformed into W303a-leu.
-pAH01-C1
-pAH02-C3
-pAH03-C2

The concentration of plasmids was about 300 ng/ul so 2ul of these solutions was added into transformation reaction.

08-08/09 Plate Reader H2O2 Induction in W303alpha

Project: Promoter
Authors: Pihla Savola
Dates: 2016-08-08 to 2016-08-09

MONDAY, 8/8

Liquid cultures of constructs

- pAH01-C1
- pAH02-C3
- pAH03-C2
- pAH04-C3
- pAH05-C10

and normal S.cerevisiae W303a (negative control) were prepared. Cells were inoculated into 5ml SD media (-leucine+2% glucose) except W303a minimal media contained all amino acids. Cells were left to grow O/N on 30 C in shaking.

TUESDAY, 8/9

Medias of pAH01-05 and W303a (NC) O/N cultures were refreshed/changed. First cells were spun down with centrifuge (5min, 3000g) and supernatant was removed. Then cells were resuspend to 5ml of fresh SD medium. With constructs pAH01-04 media was refreshed with new SD-media (- leucine + 2% glucose), with construct pAH05 media was first washed with SD-media (-leucine +2% raffinose (preparing for gal induction) and then changed to and with W303A media was refreshed to minimal meida (all amino acids +2% glucose). Cells were incubated for 5h at 30C in shaking before induction.

Before induction ODs of all refreshed cultures were balanced so that OD was around 1.0. (dillutions were made with same media what was used into refreshing).
Constructs pAH01-03 were induced with H2O2 with following H2O2 concentration mixtures. (same concentrations for each construct). Mixtures were prepaired with 30% H202. Final volumes of samples were adjusted to 1ml (sample from refressed cultures+H2O2=1ml)

Table11

	A	B	C	D	E
1	V (30 % H2O2)	C (Final concentration of H202)		Final volume (ml)	
2	0.34 uL	3 mM		1	
3	3.4 uL	30mM		1	
4	10.2 uL	0.1 M		1	
5	34 uL	0,3 M		1	
6	50.6 uL	0,5 M		1	

construct pAH05 was induced with galactose (50uL 40% galactose into 950 refressed culture=1 ml).

After induction 100uL of induced samples and W303A negative control and construct pAH04 (constituve promoter) were pipeted into 96 well plate and plate was run on plate reader with following protocol: T=30 C, time ≈20h, absorbance (600nm) and fluoresence measurent (502 nm, 528nm) every 15min and vertical shaking 731 cpm. Diagrams below contain the fluoresent values as a function of time. With first 3 diagrams fluoresence values are blanked with media and with 3 next they are blanked with negative control. All measurement data and diagrams of development of fluoresence is attached in excel file below.

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank: 2 % glucose	Negative control: W303	pJR18	pJR17								
B	Blank: 2 % raffinose											
C				pAH1 3mM	pAH1 30 mM	pAH1 0.1 M	pAH1 0.3 M	pAH1 0.5 M				
D				pAH2 3mM	pAH2 30 mM	pAH2 0.1 M	pAH2 0.3 M	pAH1 0.5 M				
E				pAH3 3mM	pAH2 30 mM	pAH3 0.1 M	pAH3 0.3 M	pAH1 0.5 M				
F												
G												
H												

OD600 and fluorescence values were blanked with the medium containing 2 % glucose and plotted as a function of time. Here for all the constructs, without H2O2:

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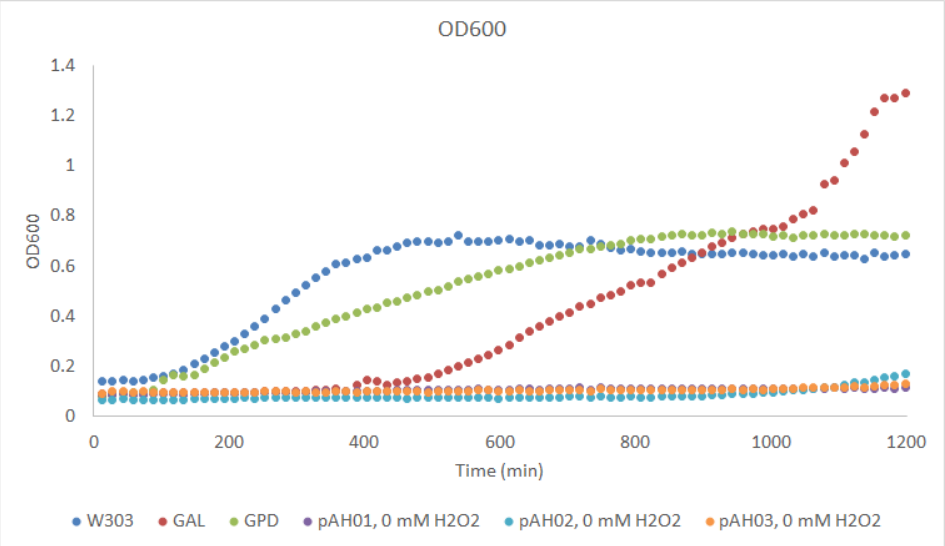

Based on this, it looks like there's been some errors in preparing the samples, as the stress promoter constructs didn't grow at all.

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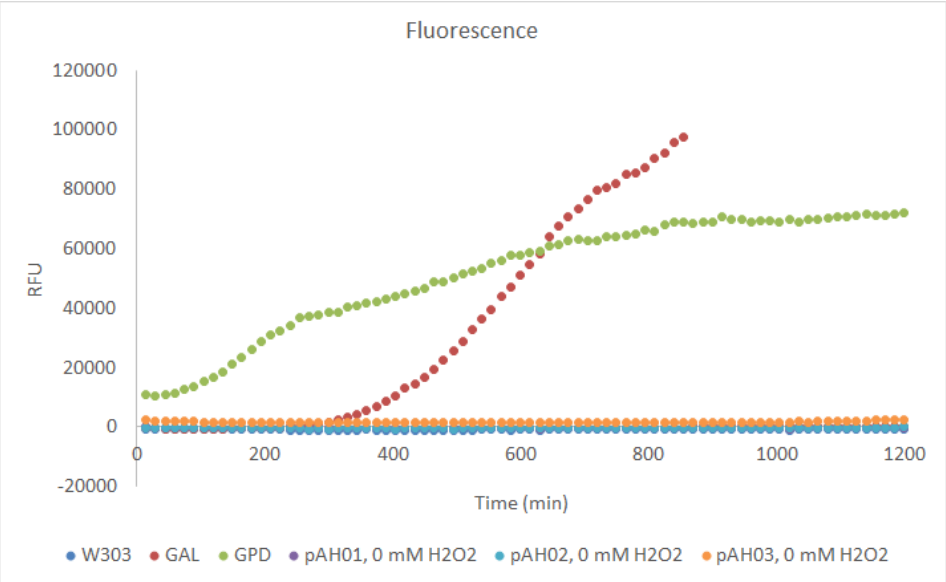

No fluorescence looks to be produced by any of the other constructs other than the control promoters, but this isn't surprising, since they didn't grow either.

Full results:

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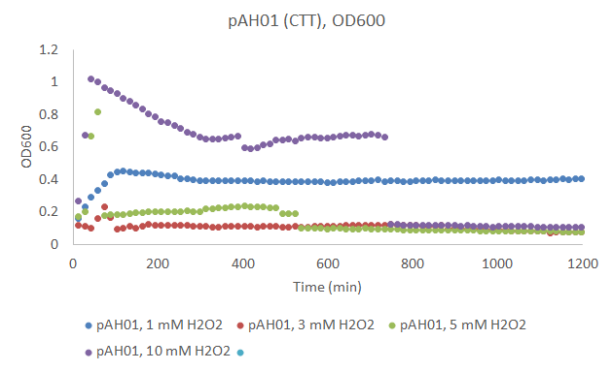

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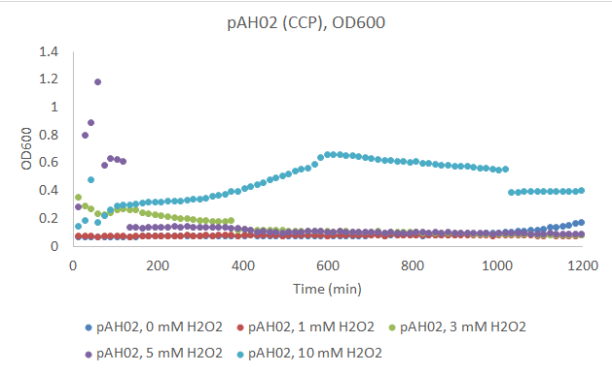

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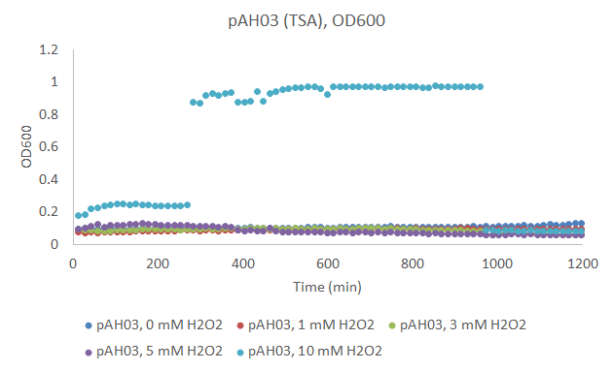

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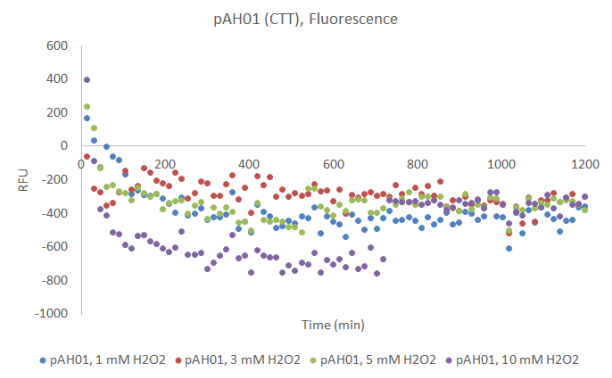

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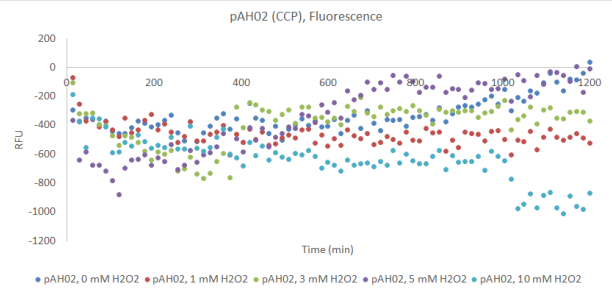

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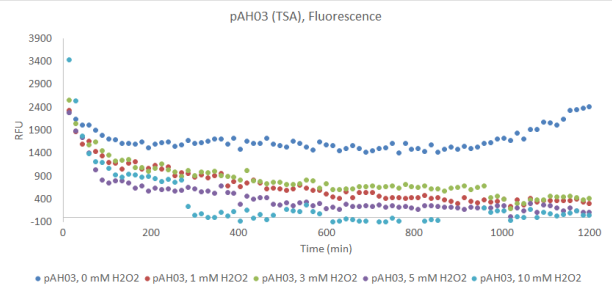

9.8 venus+stress promoters plate reader.xlsx

It was observed that fluoresence values of blank (media without cells) is very high compared to negative control and stress promoter fluoresence values which is most likely due to the high base level fluoresence of SD-media. In pAH01 and pAH02 the fluoresence of values are all the time smaller than the fluoresence of blank, but it seems they hadn't grown at all, so there has been some error in sample preparation.

Next time fluoresence measurements should be done with smaller H2O2 concentrations and blanking should be done with same construct without induction. In addition, induction should be done directly to samples in plate reader so that measurements could start sooner after induction. In addition, more frequent time points would be beneficial. Also induction with H2O2 should be done right before placing plate into plate reader in next time when stress promoters are tested.

NOTE: Later sequencing results confirmed that the promoter area in pAH02-C3 contained several point mutations, and that pAH03-C2 contained a single-base mutation in the promoter area as well as a deletion resulting in the abence of one amino acid near the C-terminus of the Venus-YFP. For this reason, different colonies were sent for sequencing for use in future experiments.

08-15/16 Retransformation to SS328-leu

Project: Promoter
Authors: Pihla Savola
Dates: 2016-08-11 to 2016-08-18

THURSDAY, 8/11

To prepare cells for transformation, *S. cerevisiae* strain SS328-leu (SS328 strain (https://www.lgcstandards-atcc.org/products/all/MYA-193.aspx?geo_country=fi) with additional deletion for leucine auxotrophy. This deletion has been selected for using nourseothricin) was streaked onto YPD plates from a glycerol stock and grown for 1 day at 30 C and 3 more days in room temperature.

MONDAY, 8/15

Liquid cultures (3x5ml) from SS328-leu were prepared in YPD. SS328-leu was also restreaked onto a new plate.

TUESDAY, 8/16

Dilutions from liquid culture were made to reach a starting OD of 0,5. Incubation in 30°C was continued.
Competent cells were done according the protocol and suspended in 200 ul of solution A. 100 ul was used for one transformation reaction.

Following constructs were transformed in SS328-leu according the LiAC transformation:

-pAH1-C1
-pAH1-C2
-pAH2-C4
-pAH3-C2
-pAH3-C3
-pAH4-C3
-pAH5-C10

All from each transformation reaction (120ul) was plated in minimal media plates.

THURSDAY, 8/18

Transformations resulted many colonies, and also single colonies could be seen.

Liquid cultures (6ml) in minimal media were prepared from following plates:

-pAH1-C1
-pAH2-C4
-pAH3-C3
-pAH4-C3
-pAH5-C10

and they were grown overnight at 30°C on shaking.

08-18/09-22 Plate Reader H2O2 induction in SS328-leu

Project: Promoter
Authors: Pihla Savola
Dates: 2016-08-18 to 2016-09-22

THURSDAY, 8/18

Liquid cultures were prepared into minimal media -leu (+2% glucose) from

- pAH01-C1
- pAH02-C4
- pAH03-C3
- pAH04-C3

in SS328-leu.

FRIDAY, 8/19

OD600s were measured and samples were diluted to reach an OD600=0,5.

Table1

	A	B	C	D	E	F
1	construct	dilution	OD600	real OD600	final dilution (to reach OD=0,5)	
2	pAH01	1:2	0,881	1,762	0,57 ml sample + 1,42 ml H2O	
3	pAH02	1	0,919	0,919	1,11+0,89	
4	pAH03	1:20	0,251	5,02	0,2+1,8	
5	pAH04	1:20	0,324	6,48	0,16+1,84	
6						

0,3% H2O2 solution was prepared from 30% stock solution.
Cells were pipetted to 96 well plate according the table 2.

Table2

	A	B	C
1	V(0,3%H2O2) ul	V(cell solution (OD=0,5)) ul	final H2O2 concentration mM
2	0	100	0
3	1,1	99	1
4	3,4	97	3
5	5,1	95	5
6	10,2	90	10

Order on 96 well plate

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	media											
B		pAH01_0	pAH01_1	pAH01_3	pAH01_5	pAH01_10						
C		pAH02_0	pAH02_1	pAH02_3	pAH02_5	pAH02_10						
D		pAH03_0	pAH03_1	pAH03_3	pAH03_5	pAH03_10						
E		pAH04_0	pAH04_1	pAH04_3	pAH04_5	pAH04_10						
F												
G												
H												

18-9 pAH01-04_ready.xlsx

For each promoter, graphs were plotted with OD600/time and, fluorescence/time. OD600 values were blanked with the medium:

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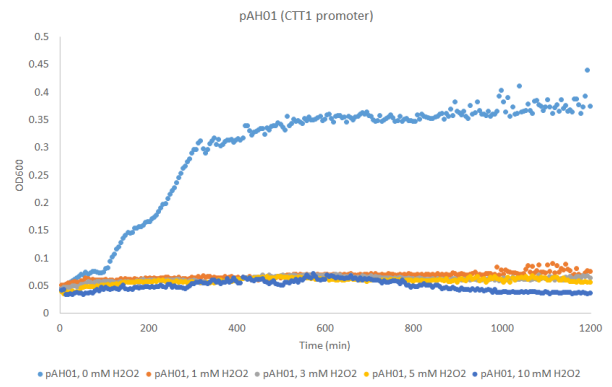

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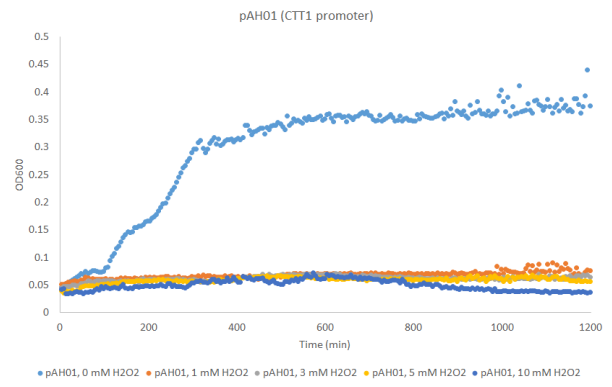
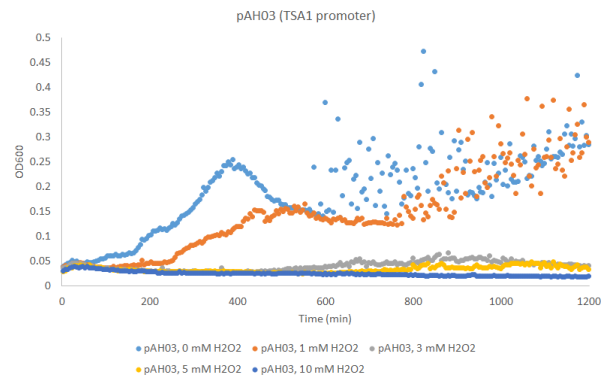


clipboard_2016-10-14_17:24:34.png



Notable that after some time, possibly cell clumping causes inaccuracy jumoing in the reads

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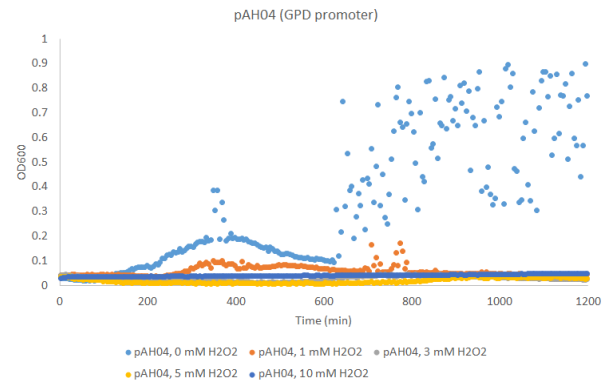

Cell clumping also here most likely, but looks like it doens't happen in every measurement.

It is seen here that the higher H2O2 concentrations slow or prevent cell growth. Fluorescence blanked with the medium:

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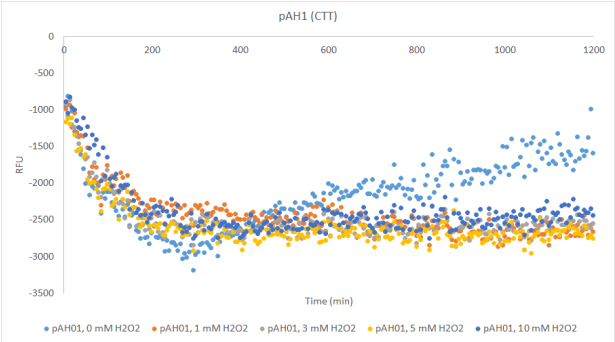

Changes in fluorescence are very small here and values smaller than media. Looks like induced values could be higher but can't really say.

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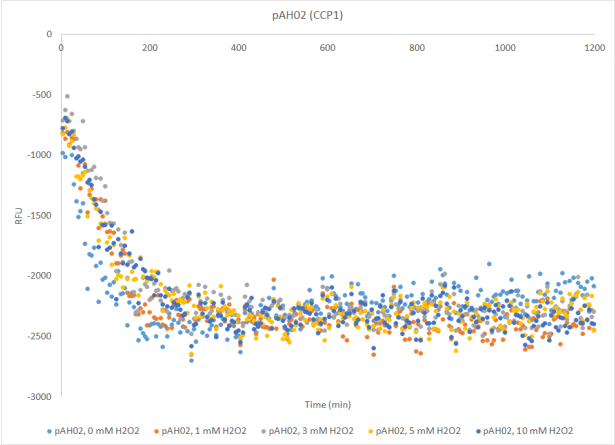

Likewise here.

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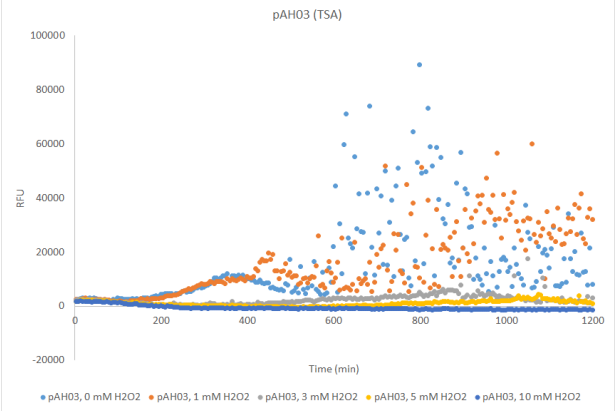

Fluorescence values are large, but get strange and jumpy around the same time as OD values, most likely cell clumping. TSA promoter looks to have a high baselevel of YFP expression, and it doesn't seem to rise with induction.

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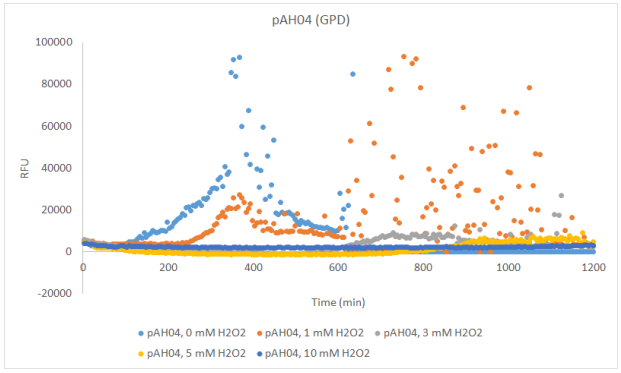

Again, cell clumping; beginning of measurement most interesting.. Looks like YFP isn't produced in higher concentrations than 1 mM H2O2

Because H2O2 has a negative effect on cell growth, it might be possible that although cell fluorescence increases with induction, this isn't observed in the raw fluorescence value - particularly in the case of pAH03/TSA. Because of this, a fluorescence/OD600 graph was plotted with blanked fluorescence and OD values:

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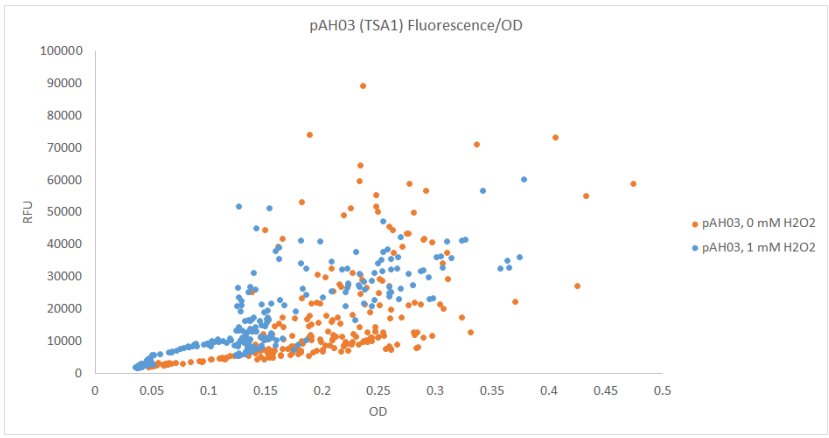

This graph looks very promising, particularly in the area of OD<0.15, which is also the area where the fluorescence and OD values didn't suffer from clumping. The experiment should be repeated to confirm these results for TSA.

In the case of CTT and CCP, the fluorescence values are too small to say anything definitive, and most likely affected by media background (see entry Venus YFP cloning)

All the experiments should be repeated with low-fluorescent media to get more reliable results. Now the background fluorescence of the media is so strong (and in the same wavelength area) that it disturbs results significantly and makes the error marginal much bigger.

NOTE: In addition, although sequencing results have confirmed pAH01-C1 and pAH03-C3 to be correct, pAH02-C4 contains two single base mutations in the promoter area. These mutations are before -300bp, so they don't affect the theoretical key transcription factor binding sites, but might nevertheless affect promoter function.

TUESDAY, 8/30

Liquid culture of constructs
pAH01-C1
pAH02-C4
pAH03-c3
pAH04-c3
pAH05-c10

and normal SS328-leu were prepared. Cells were inoculated into 5 ml of SD-media(-leu + 2 glucose) except normal SS328-leu which was inoculated into SD-media(+all amino acids + 2 % glucose). Cells were left to grow O/N at 30 C in shaking.

WEDNESDAY, 8/31

OD600s were measured from the liquid cultures pAH01-04. After measurements they were refreshed with fresh media (SD-leu+2%glu) to reach OD=0,2. Sample pAH05 was centrifuged 5min, 3900rpm and the supernatant was disgarded. Pellet was resuspended in SD-leu media with 0,5% glucose and OD was measured. Samples were diluted to OD=0,2. All tubes were left to grow for 5h in 30°C with shaking.

In the normal ss328-leu strain there was no growth. It was left anyway to grow in 30°C.

OD measurements and dilutions

Table4

	A	B	C	D	E	F
1		OD600 (1:10)	Real OD	cells: new media (ml)		
2	pAH01	0,344	3,44	0,3+4,7		
3	pAH02	0,6	6,0	0,17+4,83		
4	pAH03	0,688	6,88	0,15+4,85		
5	pAH04	0,281	2,81	0,35+4,65		
6	pAH05	0,254	2,54	0,34+4,66		
7	SS328	0,016 (no dilution)	0,016			

After refreshment samples were left to grow on 30°C with shaking for 5h.

OD measurements were repeated after this incubation.

Table5

	A	B	C	D
1		OD600 (1:10)	Real OD	cells: new media (ml)
2	pAH01	0,576	2,88	1,174+0,826
3	pAH02	0,879	4,395	0,114+0,886
4	pAH03	0,606	3,03	0,165+0,835
5	pAH04	0,972	4,86	0,103+0,9
6	pAH05	0,475	2,375	*
7				

* pAH05 is galactose inducible venus so that needed to be induced by 2% galactose. We made induction by adding 0,211 ml of cells, 100 ul of 20% galactose, 0,345 ml SD media and 0,345 ml of sterile water.

0,3% H2O2 solution was prepared from 30% stock solution.
Cells were pipetted to 96 well plate according the table 2.

Table6

	A	B	C
1	V(0,3%H2O2) ul	V(cell solution (OD=0,5)) ul	final H2O2 concentration mM
2	0	100	0
3	1,1	99	1
4	3,4	97	3
5	5,1	95	5
6	10,2	90	10

Order on 96 well plate:

Well2

	1	2	3	4	5	6	7	8	9	10	11	12
A	media (old)	media (low fluorescent)										
B		pAH01_0	pAH01_1	pAH01_3	pAH01_5	pAH01_10						
C		pAH02_0	pAH02_1	pAH02_3	pAH02_5	pAH02_10						
D		pAH03_0	pAH03_1	pAH03_3	pAH03_5	pAH03_10						
E		pAH04_0	pAH04_1	pAH04_3	pAH04_5	pAH04_10						
F		pAH05_0	pAH05_1	pAH05_3	pAH05_5	pAH010_10						
G												
H												

In the second stress promoter experiment with low-fluorescent media the OD readings were very low in the beginning. The reading was the same that in samples with only media even though the readings were calibrated with spectrophotometer to OD=0,5 the same way as last time. (Last time plate reader gave much higher readings for all the ODs.)

Anyhow the OD values in samples that were not induced with H2O2 started growing slowly meant that there must have been some cells in the beginning (except in pAH02 samples, where a pipetting error seems to have happened). Also the samples induced with 1 mM H2O2 grew slightly better than last time.

OD/Time and OD/Fluorescence graphs were drawn from fluorescence blanked with low-fluorescent media, OD600 blanked with low-fluorescent media, and time:

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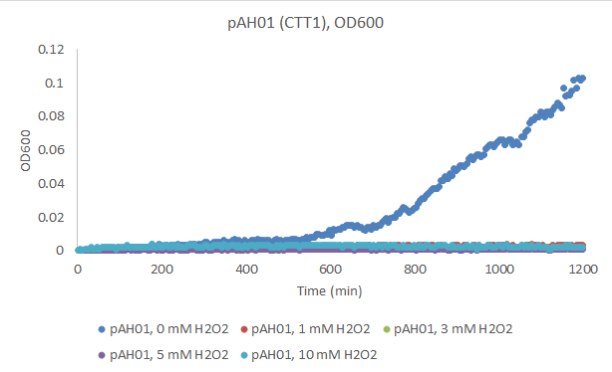
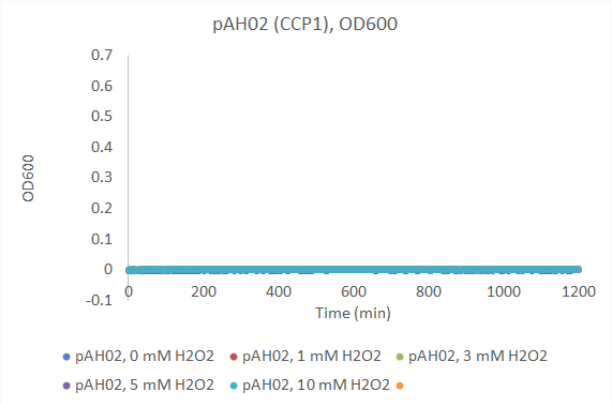
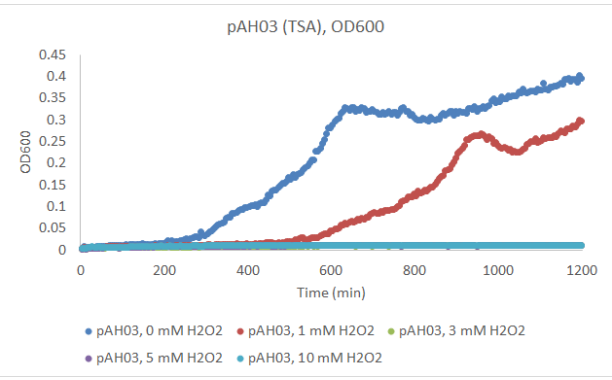


clipboard_2016-10-14_17:36:57.png

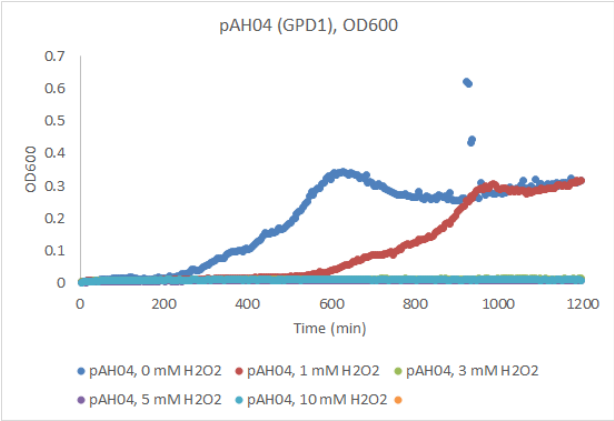


It seems there has happened a pipetting error here, since there's no growth in any of the pAH02 wells.

clipboard_2016-10-14_17:37:12.png



clipboard_2016-10-14_17:37:23.png



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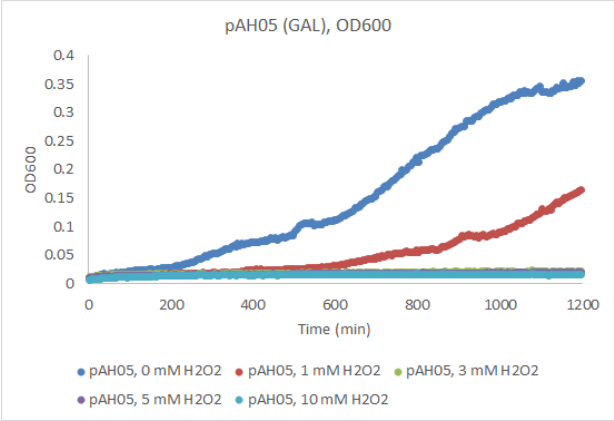
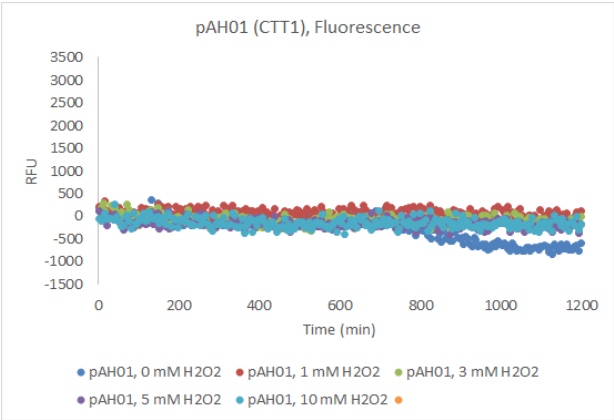


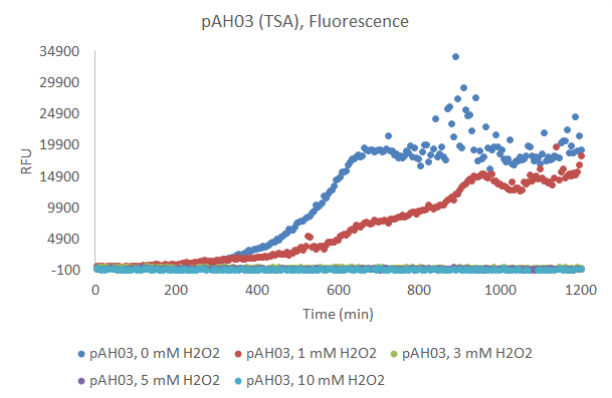
Again, in over 1 mM H2O2 the cells don't grow, and 1 mM slows growth rather consistently. Fluorescence graphs:

clipboard_2016-10-14_17:38:00.png



No fluorescence seen for pAH01.

clipboard_2016-10-14_17:38:18.png



1 mM induced fluorescence is lower than uninduced, but is the proportional fluorescence again bigger?

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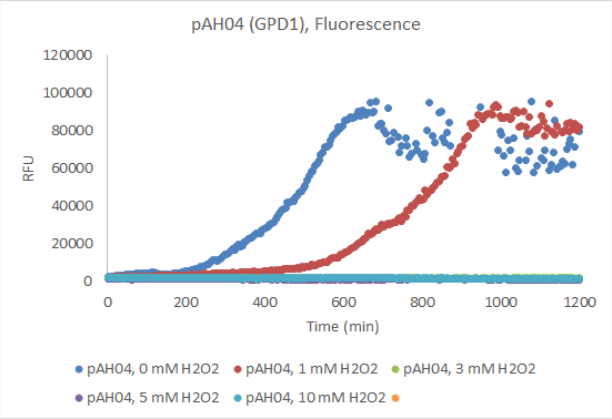

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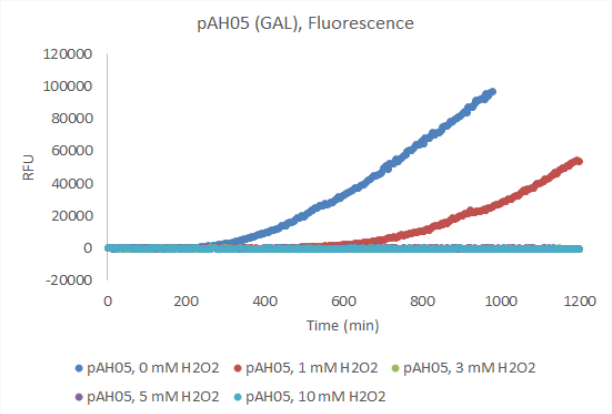
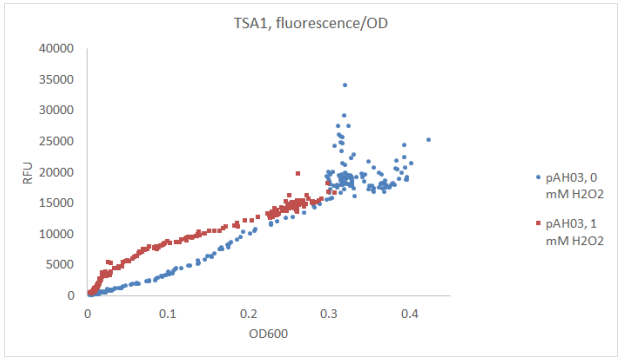


Fluorescence/OD graphs were drawn again to find out if proportional YFP production has increased. The behavior GAL and GPD was used as a comparison. Again, only 0 mM and 1 mM concentrations were plotted, since the others didn't exhibti fluorescnece or growth.

clipboard_2016-10-12_00:32:38.png



Induced TSA seems to have proportionally higher fluorescence.

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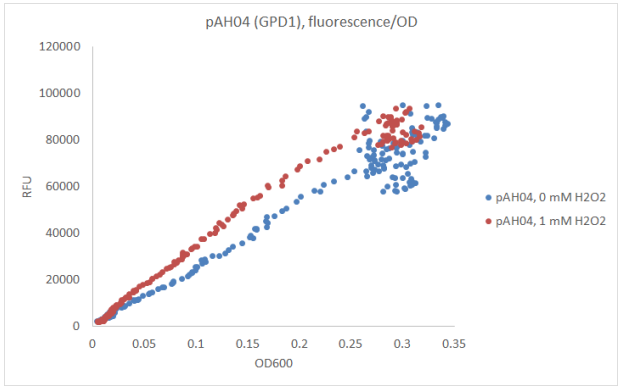

But GPD also seems to have such an increase.

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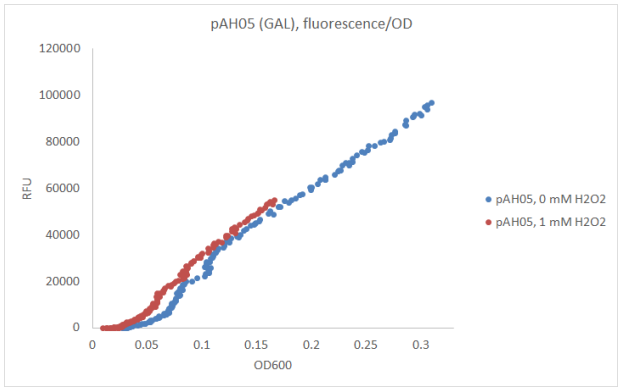

GAL, on the other hand, doedn't.

As seen in the three above graphs, it seems like TSA produces proportionally more fluorescence when induced with H2O2; but since GPD also seems to behave the same way, there's clearly also some other factors playing into this. On the other hand, the GAL promoter doesn't show such an increase; the OD/fluorescence ratio is constant between H2O2 concentrations, as it should be.

The experiment should be repeated to get data for pAH02/CCP, and to get more clarification and verification for these results. Additionally, a broader range of low H2O2 concentrations to get a better idea of the effect of H2O2.

It can also be noted that in the experiments up to now, the outgrowth has been done for too long, as the cells have reached ODs that correspond to O/N growth. It would be better to have less outgrowth, so that the cells would truly be in the phase exponential growth but not at the end of exponential/start of stationary.

Modify attachment:

31-8 pAH1-5 fluroesence measurements+induction (with low fluoresence media).xlsx

MONDAY, 9/5

SD-leu media was prepared and filter sterilized.

Liquid cultures were made from following constructs to SD-leu media:
-pAH01-C1
-pAH02-C4
-pAH03-C3
-pAH04-C3
-SS328-leu (leucine was added to this culture from 10g/L stock 15ul to 5ml)

TUESDAY, 9/6

Cells were centrifuged 3900rpm and resuspeded into new media. ODs were measured and diluted to reach OD=0,2. Leucine was again added to SS328 media. (SS328 grew lot worse than the other, and thus was not diluted but only refreshed by changing the medium.)

Table8

	A	B	C	D	E	F
1		OD600 (1:10 dilution)	OD600 real	dilution (old:new) to OD=0,2		
2	pAH01	0,177	1,77	0,56+4,44		
3	pAH02	0,387	3,85	0,26+4,74		
4	pAH03	0,959	9,59	0,10+4,90		
5	pAH04	0,337	3,37	0,29+4,71		
6	ss328-leu	0,111	0,111	no dilution		

Samples were left to grow in 30°C on shaking for 4,5h and ODs were measured again.

Table9

	A	B	C	D	E	F
1		OD600	OD600 real	dilution (old:new) to OD=0,5		
2	pAH01	0,962	0,962	0,52+0,48		
3	pAH02	0,837	0,837	0,60+0,40		
4	pAH03	0,708	0,708	0,71+0,29		
5	pAH04	1,4 (0,336 when 1:10)	1,4	0,15+0,85		
6	SS328-leu	0,432	0,432	no dilution		
7						

-0,03% H2O2 solution was prepared from 30% H2O2 stock solution.

Cells were pipetted to 96 well plate according the table 2.

Table10

	A	B	C
1	V(0,03%H2O2) ul	V(cell solution (OD=0,5)) ul	final H2O2 concentration mM
2	0	100	0
3	1,1	99	0,1
4	3,4	97	0,3
5	5,1	95	0,5
6	V(0,3%H2O2) ul		
7	1,1	99	1
8	3,4	97	3

Order on 96 well plate:

Well3

	1	2	3	4	5	6	7	8	9	10	11	12
A	media (low fluorescent)											
B		pAH01_0	pAH01_0,1	pAH01_0,3	pAH01_0,5	pAH01_1	pAH01_3					
C		pAH02_0	pAH02_0,1	pAH02_0,3	pAH02_0,5	pAH02_1	pAH02_3					
D		pAH03_0	pAH03_0,1	pAH03_0,3	pAH03_0,5	pAH03_1	pAH03_3					
E		pAH04_0	pAH04_0,1	pAH04_0,3	pAH04_0,5	pAH04_1	pAH04_3					
F		SS328_0	SS328_0,1	SS328_0,3	SS328_0,5	SS328_1	SS328_3					
G												
H												

09-07 H2O2 induction.xlsx

(Think about which pictures to get from that excel)

As before, blanked OD and fluorescence were both drawn as a function of time. OD:

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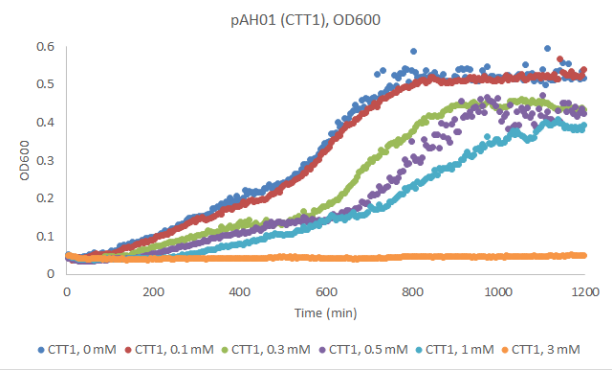
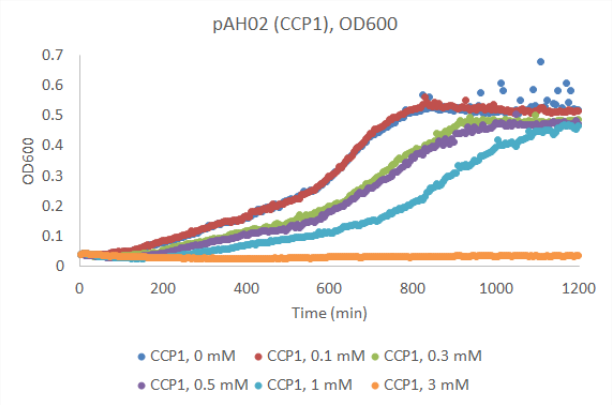
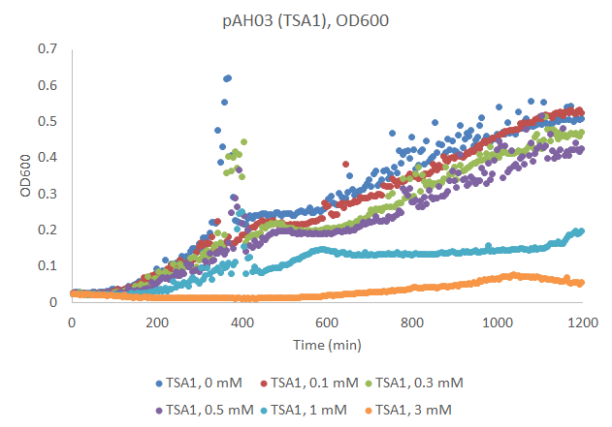


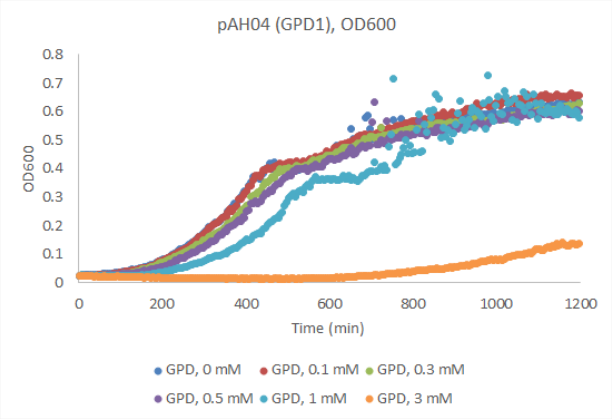
clipboard_2016-10-14_17:30:28.png



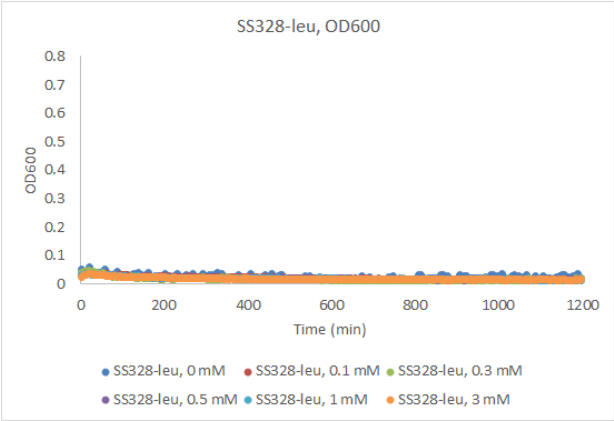
clipboard_2016-10-14_17:30:35.png



clipboard_2016-10-14_17:32:42.png



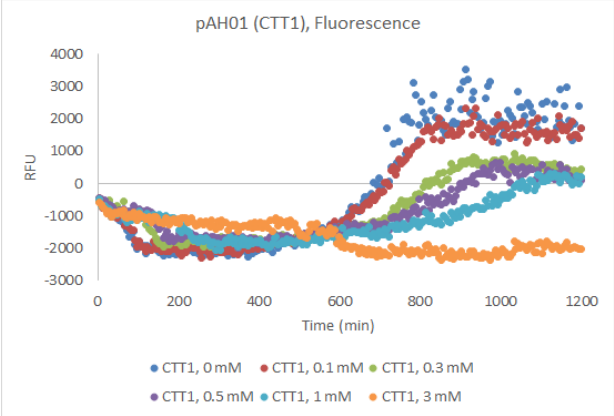
clipboard_2016-10-12_00:57:39.png



SS328-leu doesn't seem to grow on SD medium for some reason, even with supplemented leucine.

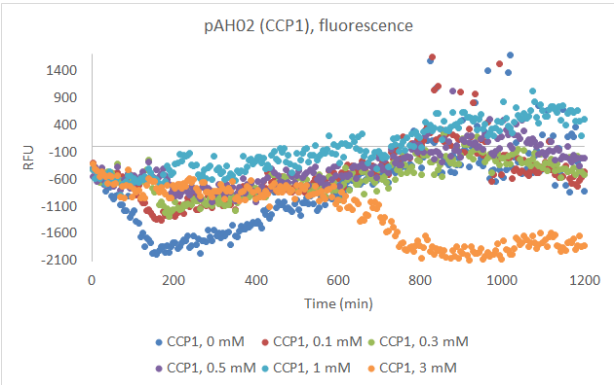
Fluorescence:

clipboard_2016-10-14_17:31:18.png



Noticable: Increase of fluorescence coincides with the time when the culture starts to reach stationary phase (refer to OD600 graph), which explains the fluorescence patterns here.

clipboard_2016-10-14_17:31:28.png



For some reason the obtained blanked fluorescence values remain negative. Still nothing conclusive for CCP.

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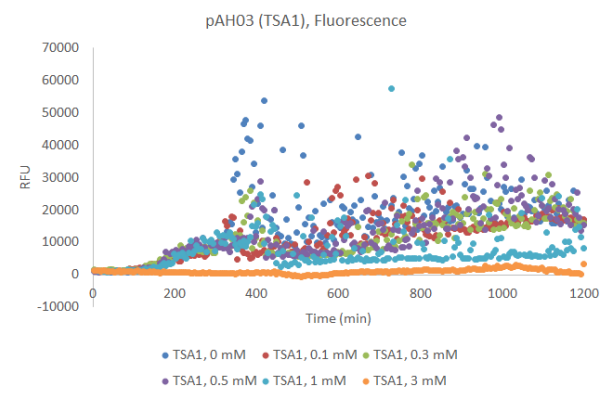

TSA looks as before.

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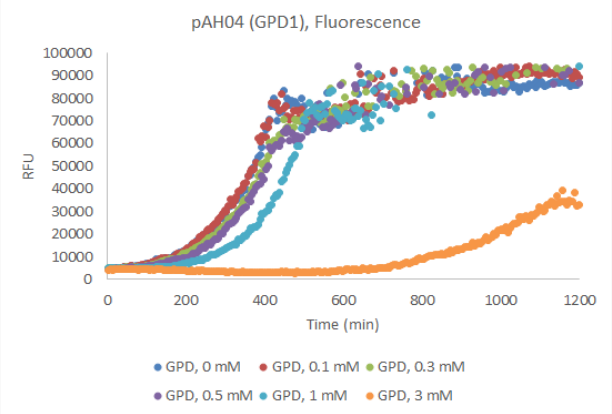

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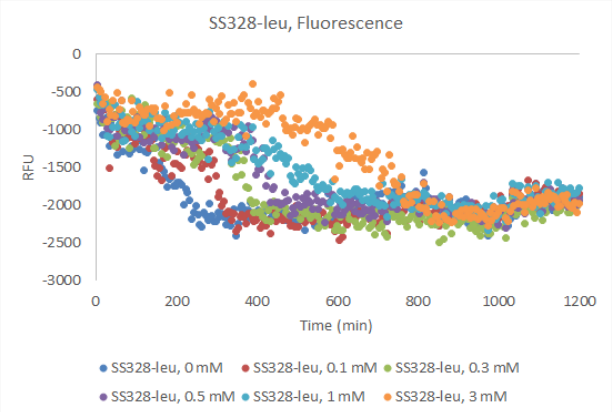

SS328-leu fluorescence values can't exavtly be compared to the others, as there was no cell growth at all.

Again, fluorescence/OD graphs were plotted:

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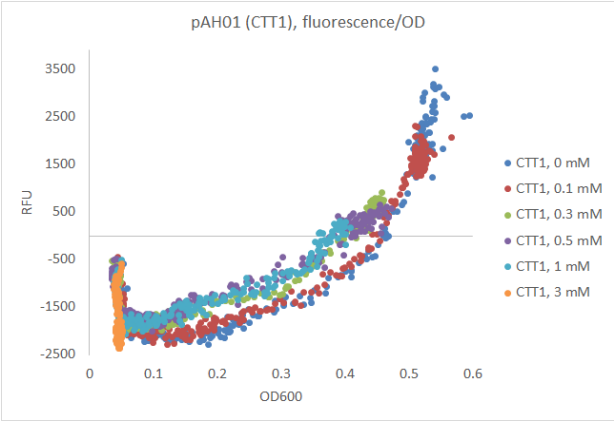
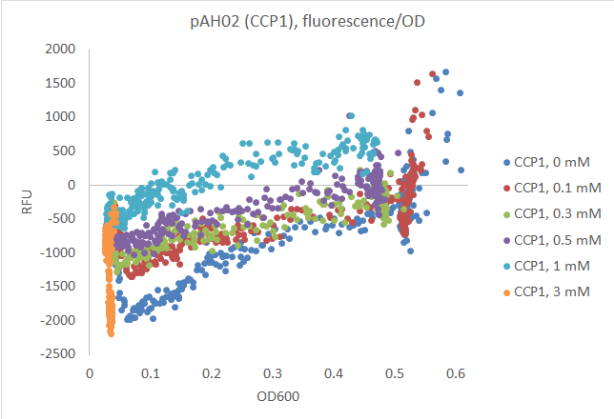
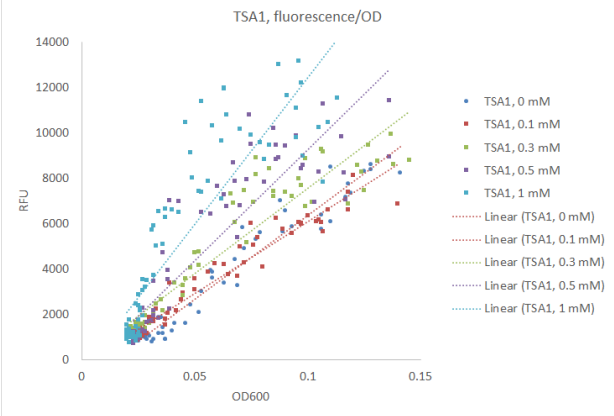


clipboard_2016-10-12_01:07:03.png

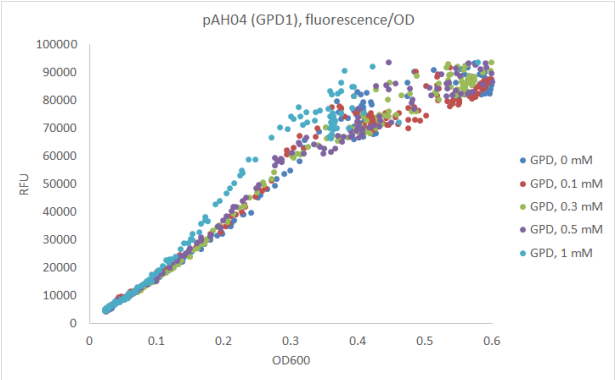


clipboard_2016-10-12_01:04:51.png



0.1 mM has been left out for clarity, and the axes have been clipped to make the picture easier to read. Cell clumping again interferes and it is difficult to say much about effect of different concentrations, although again, induced cultures seem to produce higher proportional fluorescence.

clipboard_2016-10-12_01:05:34.png



GPD promoter doesn't display as much effect of H2O2 this time, and none in the range where TSA1 already displays (lower ODs...)

Based on these data, it looks as if at least TSA1 produces a proportional increase in YFP production when exposed to H2O2, although additional verification is needed to confirm whether this apparent YFP increase is real, or simply a result of the many sources of error in this measurement.

It is clear at least, that different repeats of the experiment can produce quite different results for the same conditions. Causes for this include cell clumping, possible differences in H2O2 induction concentrations (how large a portion of the used dilution has degraded H2O2), and differences in the evening out of the measurement starting OD. In addition to this, especially in longer measurements the postion on the sample on the multiwell plate has an effect - even though the plate was sealed.

MORE on why the change could be a bias, and leadup to FACS - motivation.

08-19 pAH1-4 fluorescence+induction.xlsx

WEDNESDAY, 9/21

In addition to measuring fluorescence with flow cytometry, extra confirmation for fluorescence results was sought by redoing the microplate reader assay with triplicates, and extra attention to minimizing bias in measurements caused by sample positioning on the multiwell plate.

Overnight cultures of pAH01-4 in SS328-leu were prepared by inoculating single colonies of each construct into 5 mL volume of filter-sterilized low-fluorescent SD medium (-leu +2 % glucose). SS328-leu culture was prepared into YPD similarly.

THURSDAY, 9/22

OD values for the cultures were measured and cultures were prepared into fresh medium so that after supplementing with old culture to get an OD of 0.2, the final volume was 8 mL (pAH01) and 10 ml (pAH02-pAH04). Cultures were grown so that their OD reached about 0,5 (the ones with higher OD were diluted into OD=0,5) and then the samples were induced with hydroxen peroxide.

Dilutions:

Table13

	A	B	C	D
1		OD600 (1:5)	real OD	dilution (8ml)
2	pAH01	0,102	0,510	8ml+0ml
3	pAH02	0,112	0,560	7,2ml+0,8ml
4	pAH03	0,144	0,72	5,6ml+2,4ml
5	pAH04	0,089	0,445	8ml+0ml

These cell solution dilutions of OD ~0.5 were used to prepare induced samples for the plate reader. For the plate reader, the final volume of each sample well was calculated to be 100 uL. Each promoter was tested with three different H2O2 induction concentrations, as well as an uninduced sample, and all samples were done as triplicates.

Cell and hydrogen solutions combined for induction:

Table14

	A	B	C	D
1	final H2O2 concentration	cells	H2O2 (0,03%)	
2	0mM	100 uL	0 uL	
3	0,3mM	97 uL	3,4 uL	
4	0,6mM	93 uL	0,6 uL	
5	final H2O2 concentration	cells	H2O2 (0,3%)	
6	1,0mM	99 uL	1,1 uL	
7				

The order on the 96 well plate was as follows (letter and number markings are reversed here; in this table, A vorresponds to 1, B to 2 - and vice versa) :

Well4

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			pAH01, 1 mM	pAH01, 0.6 mM	pAH01, 0.3 mM	pAH01, 0 mM	pAH02, 0 mM	pAH02, 0.3 mM	pAH02, 0.6 mM	pAH02, 1 mM		
C		Medium	pAH01, 1 mM	pAH01, 0.6 mM	pAH01, 0.3 mM	pAH01, 0 mM	pAH02, 0 mM	pAH02, 0.3 mM	pAH02, 0.6 mM	pAH02, 1 mM		
D		Medium	pAH01, 1 mM	pAH01, 0.6 mM	pAH01, 0.3 mM	Medium	pAH02, 0 mM	pAH02, 0.3 mM	pAH02, 0.6 mM	pAH02, 1 mM		
E		Medium	pAH03, 1 mM	pAH03, 0.6 mM	pAH03, 0.3 mM	pAH03, 0 mM	pAH04, 0 mM	pAH04, 0.3 mM	pAH04, 0.6 mM	pAH04, 1 mM		
F		Medium	pAH03, 1 mM	pAH03, 0.6 mM	pAH03, 0.3 mM	pAH03, 0 mM	pAH04, 0 mM	pAH04, 0.3 mM	pAH04, 0.6 mM	pAH04, 1 mM		
G			pAH03, 1 mM	pAH03, 0.6 mM	pAH03, 0.3 mM	pAH03, 0 mM	pAH04, 0 mM	pAH04, 0.3 mM	pAH04, 0.6 mM	pAH04, 1 mM		
H												

pAH01 was only done as duplicate since there wasn't enough cell solution (part was already used for FACS samples).

For visualizing the data, averages of the replicates of each sample was used. (all triplicates raw data put somewhere too?). These averages were graphed as before.

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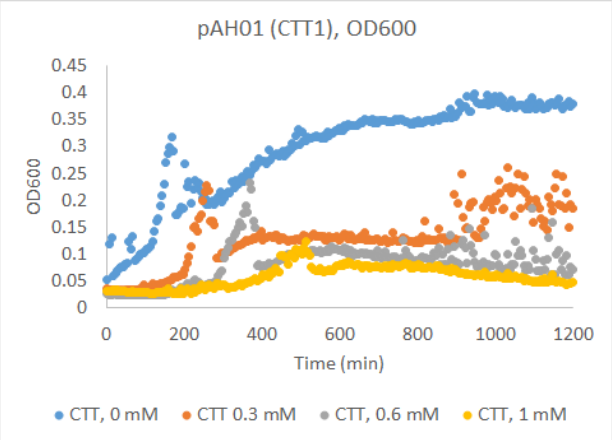
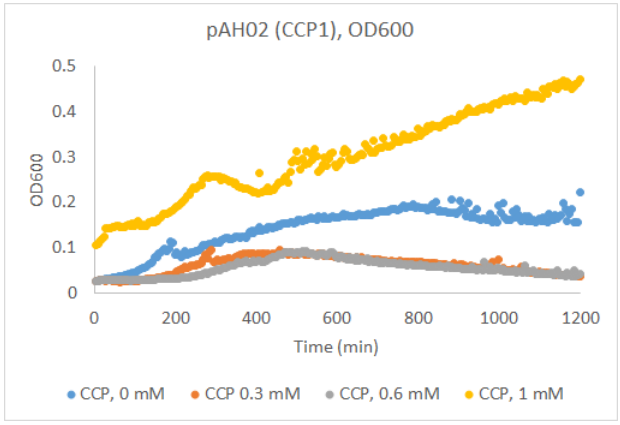


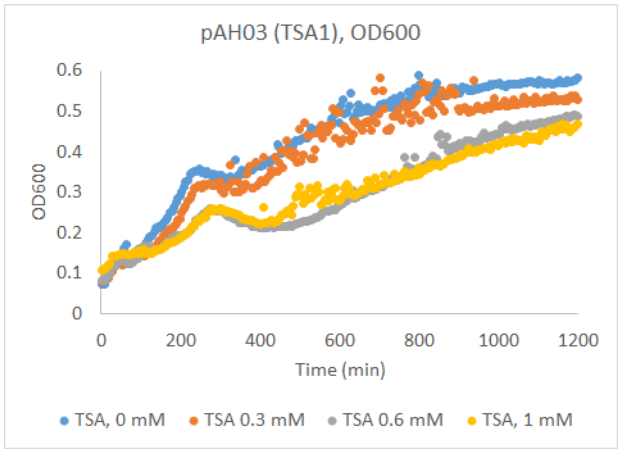
Strange "spikes" (clumping?) in all of the cultures

clipboard_2016-10-14_17:40:18.png



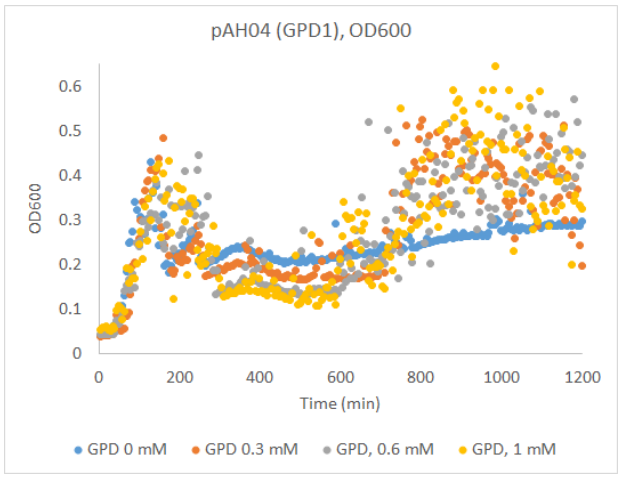
1 mM H2O2 sample has clearly had its OD evened incorrectly, as it starts out at an entirely different as the others - because of this, it is very poorly comparable to the other concentrations.

clipboard_2016-10-14_17:40:38.png



First 240 min has little deviation.

clipboard_2016-10-14_17:40:54.png



Very scattered data in all of the replicates for some reason for GPD.

Fluorescence:

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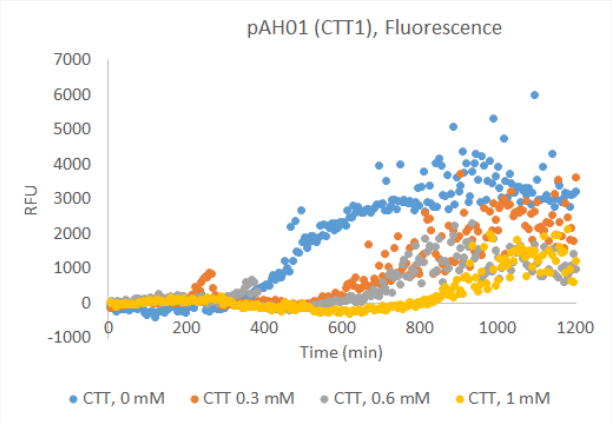
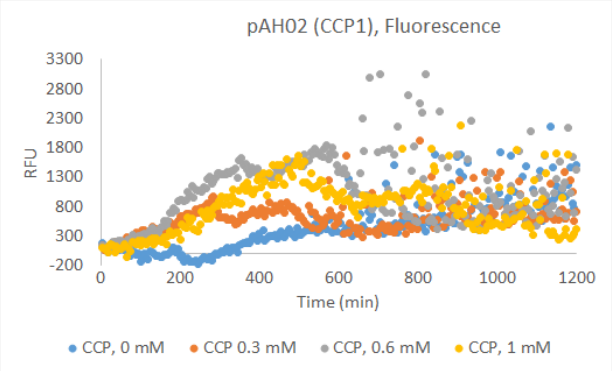
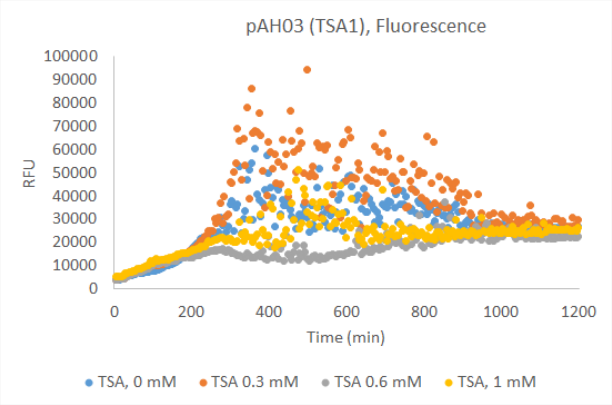


clipboard_2016-10-14_17:41:18.png



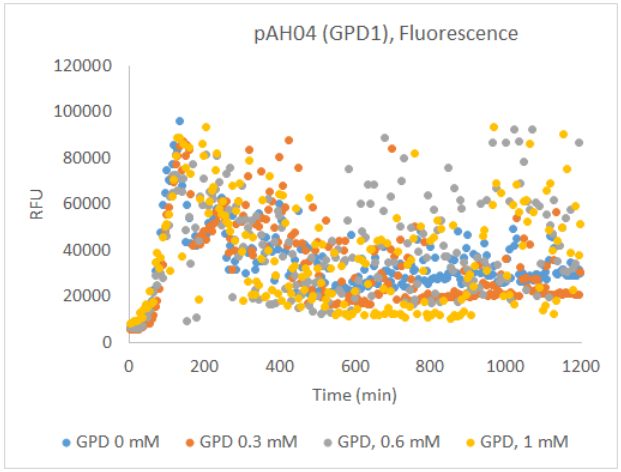
Before the read gets inaccurate, it looks like all the induction concentrations produce higher fluorescence values than uninduced, although probably because of the effect on OD, there's no clear logical relationship between concentrations.

clipboard_2016-10-14_17:42:12.png



Before 240 min, all fluorescence values are rouhgly equal, even though H2O2 slows growth.

clipboard_2016-10-14_17:42:00.png



Not much can be said of this, especially after 200 min.

Fluorescence/OD:

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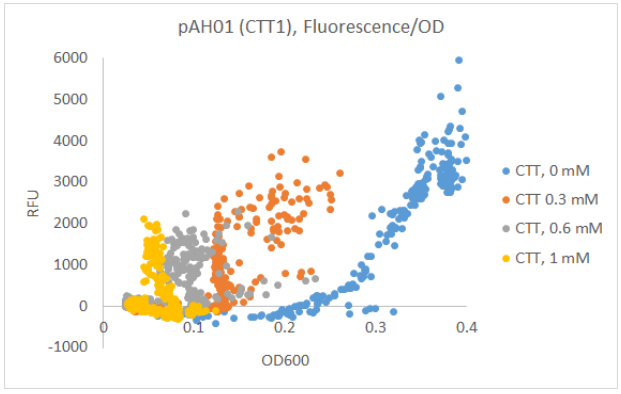
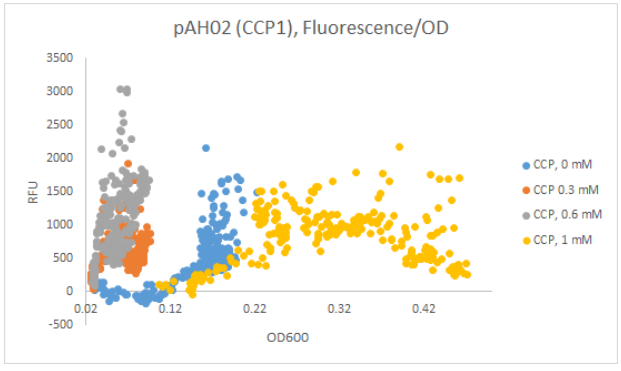
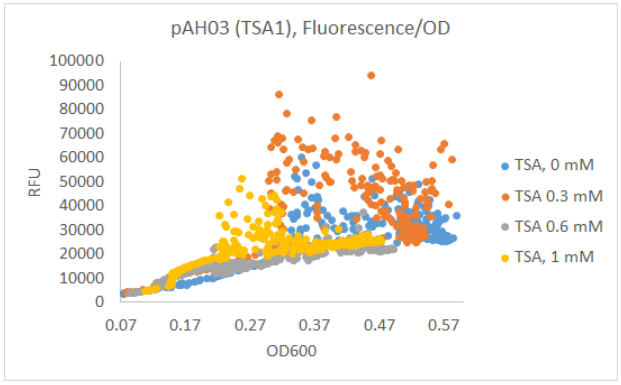


clipboard_2016-10-12_01:23:53.png



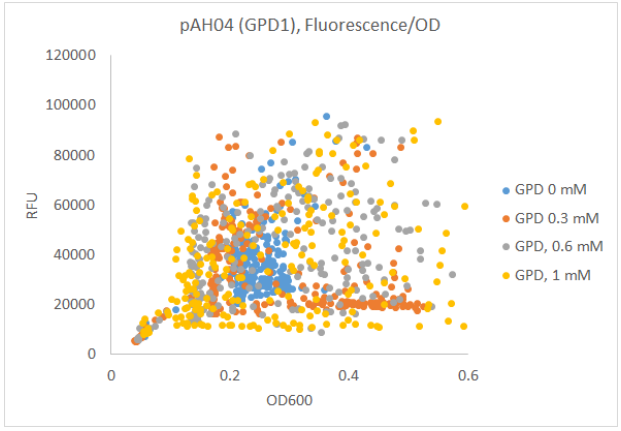
?

clipboard_2016-10-12_01:21:10.png



Without cropping the axes, it can't be seen, but in the range where OD and fluorescence wasn't funky, induction raises the fluorescence.

clipboard_2016-10-12_01:26:28.png



...

In conclusion,

blah blah these results support FACS

It is clear, however, that the tendency of yeast to clump when being grown in a microplate reader causes major probelms in measurements, as it happens sometimes and makes repeatability quite poor. Also, really the concentration of H2O2 is something that should be paid attention to.

== this assay and experiment SUCKSS

09-13/14 FACS for pAH01-4

Project: Promoter
Authors: Pihla Savola
Dates: 2016-09-13 to 2016-09-22

TUESDAY, 9/13

Overnight cultures of pAH01-4 in SS328-leu were prepared by inoculating single colonies of each construct into 5 mL volume of filter-sterilized low-fluorescent SD medium (-leu +2 % glucose). SS328-leu culture was prepared into YPD similarly.

WEDNESDAY, 9/14

OD values for the cultures were measured and cultures were prepared into fresh medium so that after supplementing with old culture to get an OD of 0.2, the final volume was 3 mL.

Resfreshed cultures were grown for 3 hours, then OD was measured and evened out to 0.37 by dilution.

Table2

	A	B	C
1		OD600	OLD+NEW (ml)
2	pAH01	0,488	3,8ml+1,2ml
3	pAH02	0,530	3,5ml+1,5ml
4	pAH03	0,384	4,8ml+0,2ml
5	pAH04	0,371	5ml+0ml

For induction with H2O2, culture and 0.3 % H2O2 solution were combined to acheive a total volume of 1 mL:

Table3

	A	B	C
1	0,3% H2O2	cells	
2	0	1000	
3	3,4	1000	
4	5,1	1000	
5	10,2	990	
6	30,10	900 (note that total volume is different here!)	

Induced samples were incubated at 30 C for 1 hour. OD values were measured; values were in the range of 0.3-0.4.

Final samples for measurement with FACS were done by combining culture medium and PBS so that the final OD would be ~ 0.1; 300 uL of culture and 700 uL PBS were combined.

Samples were analyzed with FACS with the following parameters; FSC 571, SSC 319, FITC 890 (for the first sample, 390,319,676 was tried at first but then this was changed.)

The naming of the sample results was as follows:

Table1

	A	B
1	Sample 1	pAH04-C3, 0 mM
2	Sample 2	pAH04-C3, 0.3 mM
3	Sample 3	pAH04-C3, 0.5 mM
4	Sample 4	pAH04-C3, 1 mM
5	Sample 5	pAH04-C3, 3 mM
6	Sample 6	pAH02-C4, 0 mM
7	Sample 7	pAH02-C4, 0.3 mM
8	Sample 8	pAH02-C4, 0.5 mM
9	Sample 9	pAH02-C4, 1 mM
10	Sample 10	pAH02-C4, 3 mM
11	Sample 11	pAH01-C1, 0 mM
12	Sample 12	pAH01-C1, 0.3 mM
13	Sample 13	pAH01-C1, 0.5 mM
14	Sample 14	pAH01-C1, 1 mM
15	Sample 15	pAH01-C1, 3 mM
16	Sample 16	pAH03-C3, 0 mM
17	Sample 17	pAH03-C3, 0.3 mM
18	Sample 18	pAH03-C3, 0.5 mM
19	Sample 19	pAH03-C3, 1 mM
20	Sample 20	pAH03-C3, 3 mM
21	Sample 21	SD medium

However, no substantial differences were observed between any of the samples. There wasn't time to try other measurement parameters, but most likely something was wrong with them.

pAH04 (GPD promoter), at the very least, should have shown considerably more fluorescence than the others, as has been seen in plate reader results. The others proably would have needed higher induction times for visible results.

Results are attached below.

Blank Experiment with Sample Tube_004-Batch_Analysis_29092016130159.pdf

Overnight cultures of pAH01-4 in SS328-leu were prepared by inoculating single colonies of each construct into 5 mL volume of filter-sterilized low-fluorescent SD medium (-leu +2 % glucose). SS328-leu culture was prepared into YPD similarly.

THURSDAY, 9/22

OD values for the cultures were measured and cultures were prepared into fresh medium so that after supplementing with old culture to get an OD of 0.2, the final volume was 8 mL (pAH01) and 10 ml (pAH02-pAH04). Cultures were grown so that their OD reached about 0,5 (the ones with higher OD were diluted into OD=0,5) and then the samples were induced with hydroxen peroxide.

Dilutions:

Table4

	A	B	C	D
1		OD600 (1:5)	real OD	dilution (8ml)
2	pAH01	0,102	0,510	8ml+0ml
3	pAH02	0,112	0,560	7,2ml+0,8ml
4	pAH03	0,144	0,72	5,6ml+2,4ml
5	pAH04	0,089	0,445	8ml+0ml

Induction:

Table5

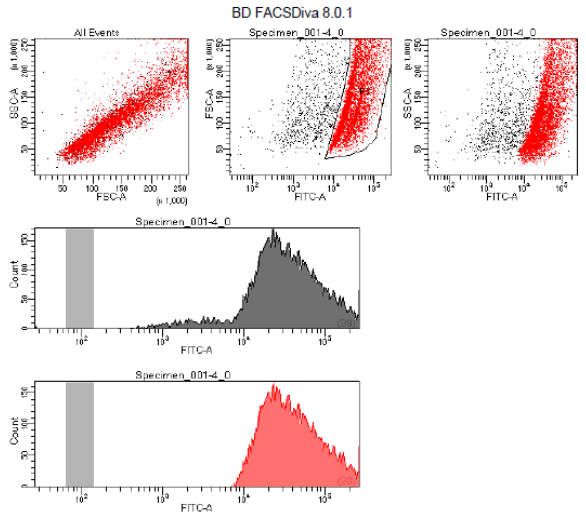
	A	B	C	D
1	final H2O2 concentration	cells	H2O2 (0,3%)	
2	0mM	1500	0,0	
3	0,3mM	1500	5,1	
4	0,6mM	1500	10,2	
5	1,0mM	1500	15,3	

Induced samples were analysed 2h and 4h after the induction. Samples for FACS were prepared so that PBS and cell culture were combined to a final volume of 1 mL, with an OD was about 0,1 in the measurement. After 2h incubation this meant dilutions 1:10 - 1:5 from the induced culture. The same was done at the 4 h timepoint. Since we used low-fluorescent media in our experiment, the remaining media in the cell culture shouldn't affect the results.

Measurements were done with BDFACSAria III. The used parameters were: FSC 43, SSC 311, FITC 392 (FITC scaled as logarithmic). A threshold of 10 000 events was used for the measurement, with flow rate 4.

An example of obtained uncprocessed results:

clipboard_2016-10-12_14:45:21.png



(Value gating (here: red) wasn't used in further data processing, but done here out of curiosity)

Full report attached below:

Blank Experiment with Sample Tube_005-Batch_ Analysis_29092016131142.pdf

Median fluorescence was decided to be used as the fluorescence values weren't normally distributed. The following median fluorescence values were obtained at the 2 h measurement:

Table6

	A	B	C	D	E	F
1	H2O2 (mM)	GPD1	TSA1	CCP1	CTT1	SS328-leu
2	0	32325	11551	1334	730	70
3	0.3	35880	16824	5654	2191	
4	0.6	40994	22545	4131	2260	
5	1	43757	24420	5961	1275	

For 4 h:

Table7

	A	B	C	D	E
1	H2O2 (mM)	GPD1	TSA1	CCP1	CTT1
2	0	32673	12792	1084	767
3	0.3	33650	14149	4015	1856
4	0.6	32802	16888	8257	1736
5	1	41687	21952	5675	1651

Fluorescence values were graphed for both time points as a function of H2O2 concentration:

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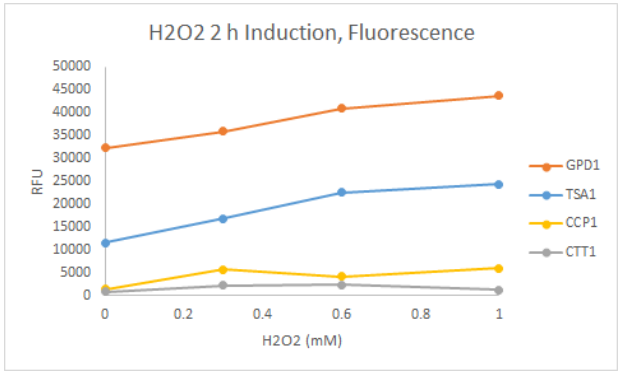
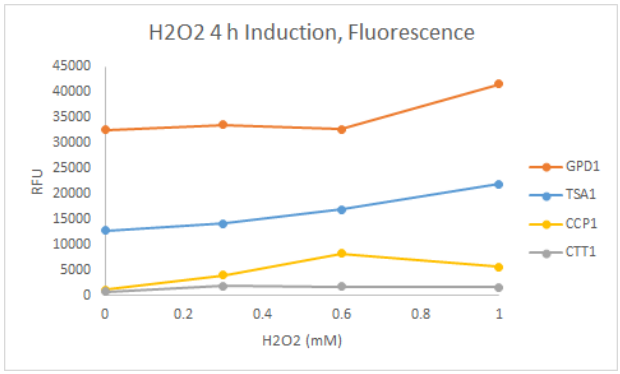


clipboard_2016-10-12_15:47:39.png



This gives an idea of the general expression levels of YFP, but changes in fluorescence can't be compared easily, because when the values are big to begin with, changes that are percentually small look very big in comparison. To account for that bias, fluorescence values were normalized to uninduced fluorescence values :

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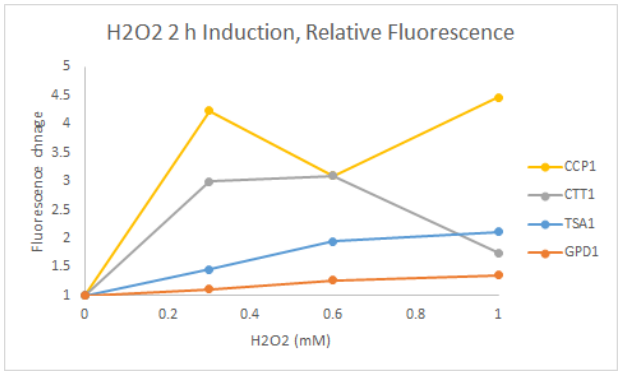
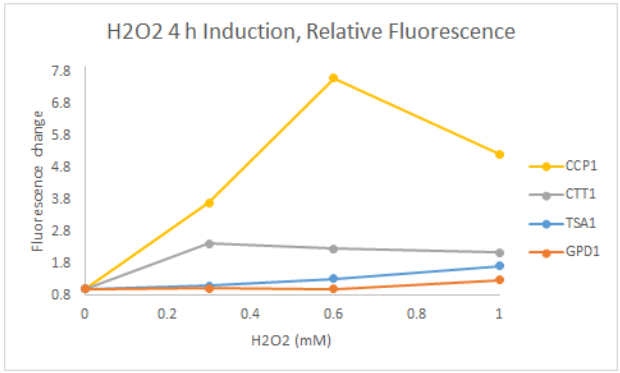


clipboard_2016-10-12_15:53:07.png



With normalization, it's clear that although the fluorescence of the GPD promoter increases with H2O2 concentration, the changes are much smaller than in the case of the stress promoter constructs. This seeming increase could e.g. be caused by the fact that H2O2 slows cell culutre growth, meaning that a relatively larger portion of measured cells are older and have had more time to accumulate fluorescence, leading to elevated readings.

In the case of CCP and CTT, the intitial expression level is very low, which explains some of the jumpiness in the relative fluorescence: when the values are small, smaller shifts turn out as bumps. Indeed, more measurements would be needed to better characterize the pattern of H2O2 effect on these promoters.

All the same, changes in relative fluorescence are so substantial, that even though mor measurements would provide accuracy and proper characterization, these changes are very unlikely to be due to just background factors. Therefore, based on this, all the promoters seem to be working. CCP1 promoter produces the biggest change in expression when induced, which could indicate that if the expression change signal was amplified, it could produce a very pronounced difference in response to stress.

Still, as these test have been done with H2O2 induction, actual MC induction would be needed to confirm if the promoters respod as well to the actual toxin.

10/03-11 Promoter MC induction + FACS

Project: Promoter
Authors: Pihla Savola
Dates: 2016-10-03 to 2016-10-11

MONDAY, 10/3

This entry describes induction of stress promoters with MC, and measurement of the fluorescence with FACS.

Two 5 mL precultures of promoter constructs (pAH01-C1, pAH02-C4, pAH03-C3 and pAH04-C3) in SS328-leu+pAH09 were prepared from plates transformed on 09/14-15. Single coloneis were inoculated from the plates into SD-medium-leu. Growth was at 30 C with shaking.

TUESDAY, 10/4

No growth was visible in any of the cultures on the following morning or even later in the day, so th MC induction had to be postponed.

WEDNESDAY, 10/5

All transporter+promoter transformation plates were restreaked on fresh SD-leu agar plates. Also, to test the medium which was used earlier this week, 3 mL cultures were inoculated with colonies and grown overnight with shaking at 30 C. The following cultures were prepared:

- pAH03-C3 in SS328-leu+pAH9
- pAH03-C3 in SS328-leu

This time, more cells were picked for inoculation than last time just to be sure (the colonies on the plate were quite small)

THURSDAY, 10/6

OD was measured in the morning:

- pAH03-C3 in SS328-leu+pAH9 - OD 0.7
- pAH03-C3 in SS328-leu - OD 7

Based on this, it looks like the medium is OK, and when inoculating enough from the fresh plates, there shouldn't be further problems.

FRIDAY, 10/7

Restreaked plates were collected and stored in +4 C.

MONDAY, 10/10

The following overnight precultures were prepared by inoculating single colonies from the new plates into 5 mL SD-medium -leu:

- pAH01-C1 in SS328-leu
- pAH01-C1 in SS328-leu+pAH09-C2-C5
- pAH02-C4 in SS328-leu
- pAH02-C4 in SS328-leu+pAH09-C2-C5
- pAH03-C3 in SS328-leu
- pAH03-C3 in SS328-leu+pAH09-C2-C5
- pAH04-C3 in SS328-leu
- pAH04-C3 in SS328-leu+pAH09-C2-C5

In addition, a 5 mL preculture of SS328-leu was also prepared, but into YPD.
Cultures were grown overnight at 30 C, 240 rpm.

TUESDAY, 10/11

OD's of the cultures were measured, and calculations were made for how much preculture and fresh SD medium would have to be combined to reach an OD of ~0.3 in a total volume of 10 mL

Table1

	A	B	C	D	E
1	Culture	OD, 1:10	Real OD	Preculture for new culture (mL)	Fresh medium fo new culture (mL)
2	pAH01-C1 in SS328-leu	0.556	5.56	0.5	9
3	pAH01-C1 in SS328-leu+pAH09-C2-C5	-	0.608	4	
4	pAH02-C4 in SS328-leu	0.629	6.29	0.5	9
5	pAH02-C4 in SS328-leu+pAH09-C2-C5	0.149	1.49	2	
6	pAH03-C3 in SS328-leu	0.647	6.47	0.5	9
7	pAH03-C3 in SS328-leu+pAH09-C2-C5	0.318	3.18	1	
8	pAH04-C3 in SS328-leu	0.687	6.87	0.5	9
9	pAH04-C3 in SS328-leu+pAH09-C2-C5	0.478	4.78	0.6	9

As seen in the table, for pAH01-C1 in SS328-leu+pAH09-C2-C5 the OD was so low that only a total volume of 8 mL was reached. In the case of pAH01-C1 in SS328-leu+pAH09-C2-C5 and pAH02-C4 in SS328-leu+pAH09-C2-C5, because the ODs were so low, and refreshed cultures would have had so little fresh medium, the preculture volume indicated in the table was spun down (3000 rpm, 5 min), old medium was removed, and pellet was resuspended in the desired volume (8 mL/10mL) of fresh medium.

New cultures were prepared in 250 mL flasks and grown at 30C, 240 rpm for 6 hours. (ODs were measured already after 4.5h incubation but they were not high enough for incubation, as lowest ODs were only 0.3. For this reason we decided to continue incubation for an additional 1,5hours.)

The SS328-leu preculture had OD 8, and was refreshed by combining 0.2 mL old culture with 4.8 mL new culture, and left to grow in 30 C, 240 rpm.

For induction, ODs of the cultures were measured:

Table2

	A	B	C
1	Culture	REAL OD	
2	pAH01-C1 in SS328-leu	-	
3	pAH01-C1 in SS328-leu+pAH09-C2-C5	0.285	
4	pAH02-C4 in SS328-leu	0.956	
5	pAH02-C4 in SS328-leu+pAH09-C2-C5	0.691	
6	pAH03-C3 in SS328-leu	1.436	
7	pAH03-C3 in SS328-leu+pAH09-C2-C5	0.914	
8	pAH04-C3 in SS328-leu	1.234	
9	pAH04-C3 in SS328-leu+pAH09-C2-C5	1.136	

pAH01-C1 in SS328-leu+pAH09-C2-C5 had not grown at all after refreshing so we decided not to use it in our experimets. For this reason also pAH01-C1 became unnecessary as it was only a control for pAH01-C1 in SS328-leu+pAH09-C2-C5.

For MC induction, MC dilutions were prepared so that they would have 10x concentration compared to the desired induction concentration. Filter-sterilized 50 ug/mL extract was used. Glass pipettes were used in handling all solutions containing MC that wasn't the original extract.

Table4

	A	B	C	D	E
1	Prepared MC dilution (ug/L)	Induction concentration to use it for (ug/L)	MC extract (uL)	H2O (uL)	Previous (more concentrated) dilution (uL)
2	1000	100	40	1960	-
3	100	10	-	1800	200
4	10	1	-	1800	200

Final induction cultures were prepared as follows in sterilized glass test tubes:

Table3

	A	B	C	D	E	F	G
1		pAH02-C4 in SS328-leu	pAH02-C4 in SS328-leu+pAH09-C2-C5	pAH03-C3 in SS328-leu	pAH03-C3 in SS328-leu+pAH09-C2-C5	pAH04-C3 in SS328-leu	pAH04-C3 in SS328-leu+pAH09-C2-C5
2	Old culture (mL)	1.05	1.45	0.7	1.09	0.81	0.88
3	MC solution (mL) / water for c(MC)=OmM	0.2	0.2	0.2	0.2	0.2	0.2
4	Fresh medium (mL)	0.95	0.55	1.3	0.91	1.19	1.12
5	total (mL)	2.2	2.2	2.2	2.2	2.2	2.2

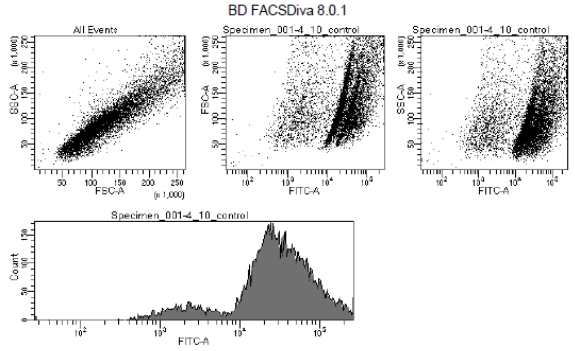
Induced culture were left to grow for 2 hours at 30°C with shaking.

After induction, cultures were spun down at 3000 rpm, 10 min. 2 mL of the SS328-leu culture in YPD was also spun down. Supernatant was discarded and pellets were resuspended in 2 mL of PBS. OD's of some of the resuspended cultures were measured; they were between 0.7-1.2. Based on this, 100 uL of the the cell-PBS suspension was combined with 1 mL of PBS to prepare the final samples for the flow cytometer.

Measurements were done with BDFACSAria III. The same parameters as used for H2O2 were suitable also here (FSC 43, SSC 311, FITC 392 - FITC scaled as logarithmic). A threshold of 10 000 events was used for the measurement, with flow rate 4.

An example of obtained uncprocessed results:

clipboard_2016-10-12_14:42:31.png



Median fluorescence was decided to be used as the fluorescence values weren't normally distributed. The following median fluorescence values were obtained:

Table5

	A	B	C	D	E	F	G	H
1	MC (ug/L)	GPD1 + Transporter	GPD1 w/o Transporter	TSA1 + Transporter	TSA1 w/o Transporter	CCP1 + Transporter	CCP1 w/o Transporter	SS328-leu
2	0	48939	29961	11823	7974	874	1069	67
3	1	52709	30552	9643	7827	871	964	
4	10	53102	28455	9642	7733	862	1064	
5	100	47005	26627	11105	8216	889	992	

Full report of results in the attached file:

iGEM MC induction-Batch_Analysis_11102016205040.pdf

The fluorescence values were plotted as a function of MC concentration:

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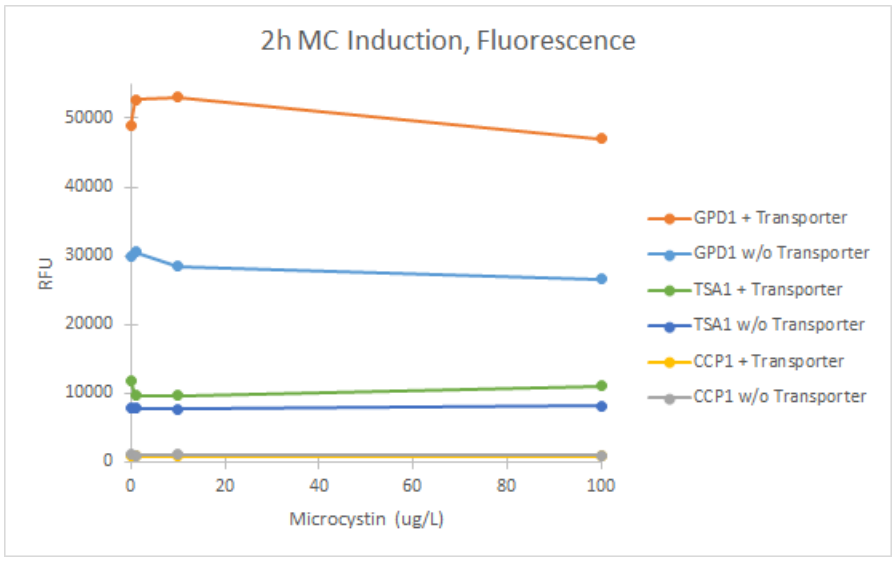

It is clear that all promoters produce fluorescence compared to the negative control, but it looks like MC concentration doesn't have any effect. For some reason, particularly with GPD, there's a huge difference between transporter/no-transporter strains, although within the strain, fluorescence remains constant. A possible explanation for this might be that the change of transporter has some unforeseen effects on cell metabolism. Some differences can perhaps also be explained by how the different strains grew at diffrent rates, and in cultures at different stages of growth have differences in how much fluorescence an individual cell has accumulated inside.

Based on these results, more verification would be needed on whether the integrated transporter is functional; previous tests have been unable to obtain consistent data on the effect of microcystin on cells, even the positive control (VL3). (something about different transporter variants?)

Another possibility, although not perhaps as likely, is that the MC in the extract was degraded, as this has happened to us before. The extract has been handled properly, though, so this doesn't seem likely.